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ENDOCRINOLOGY

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THE EFFECT OF THIOURACIL HYPOTHYROIDISM ON REPRODUCTION IN THE RAT

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IT HAS long been assumed that there is some relationship between thyroid activity and the reproductive system. Although much experimental work has been done in an effort to elucidate the problem, results have been conflicting. Most of the literature deals with the histologic and metabolic effects of thyroidectomy on special tissues, the effect on the estrus cycle as reflected in vaginal smears, or the physiological effect on the pituitary gland as judged by extraction or implantation experiments. Direct mating experiments are rare. The technical difficulties associated with thyroidectomy contribute largely to the confusion of results as the regenerative powers of the thyroid gland are so great that even the presence of a few thyroid cells after operation may alter the experimental findings. With the advent of thiouracil and related compounds a method was offered for the study of hypothyroidism which obviated the technical difficulties and sources of error associated with thyroid ablation. Using the thiouracil technique, a series of experiments were planned in an effort to demonstrate what specific effect hypothyroidism might exert on the ability of rats to reproduce.

Experimental: A group of 14 adult male rats were first studied. These rats had as their only source of fluid a 0.1 per cent solution of thiouracil, making the calculated dosage of thiouracil approximately 24 mgm. a day per animal which is well above the amount necessary to produce hypothyroidism in rats (Ref. 1). Certain groups of animals were mated after having been fed thiouracil over periods of 49, 166, 204 and 259 days. Each male was mated with a single female and the

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thiouracil water bottles were removed each night when the female was placed in the mating cage. The results of these matings can be seen in Chart 1.

Of 6 males mated 49 days after the beginning of thiouracil administration 6 sired litters, 5 of which progressed to term normally and one of which resorbed. Animals 1 and 2 were sacrificed. The thyroid glands weighed 39.8 and 56.0 mgm. in contrast to glands of control animals which averaged 19 mgm. Histologically the glands were markedly hyperplastic. The weight and appearance of the testes were normal. Four of these rats and 2 additional ones were mated on the 166th day of thiouracil administration; 3 sired normal litters including rat No. 3 whose previous mating had resulted in a resorption. The

CHART 1. RESULTS OF MATINGS OF MALE RATS FED A 0.1% SOLUTION OF THIOURACIL

Rat #	1st mating group			2nd mating group			3rd mating group			4th mating group			Day of autopsy
	Days on drug	Day of del.	# in litter	Days on drug	Day of del.	# in litter	Days on drug	Day of del.	# in litter	Days on drug	Day of del.	# in litter	
1	49	33	4										60
2	49	40	6										59
3	49	31	resorp.	166	23	6							211
4	49	33	8	166	30	8							211
5	49	40	5	166	24	4							211
6	49	35	8	166	—	—	204	26	7				257
7				166	—	—	204	34	5				257
8				166	—	—	204	—	—	259	—	—	335 Lympho-sarcoma
9							204	—	—				251 Died
10										259	—	—	340
11										259	25	11	327
12										259	57	7	327
13										259	—	—	280 Died
14										259	94	4	340

remaining 3 animals failed to impregnate the females during a period of one month. These 3 rats and an additional one, No. 9, were remated on the 204th day. Two sired normal litters while the mating of No. 8 was again unfertile. Rat No. 9 also had a sterile mating but, soon after the removal of the female, the male appeared to be ill and died. At autopsy there was a marked reduction in body weight, 234 gm., in contrast to the average weight of 352.6 gm. for treated animals, the thyroid gland weighed only 50 mgm. as compared with the average weight of 101.5 mgm, and the adrenals weighed 74 mgm. against the average of 26 mgm. Blood and bone marrow studies were not made and sections were not taken because of autolization. Rat No. 8 was remated for the third time on day 259 as were the last 5 animals in the series. Of these matings 3 were sterile (rats 8, 9, and 13), and 3 resulted in normal litters. However the duration between

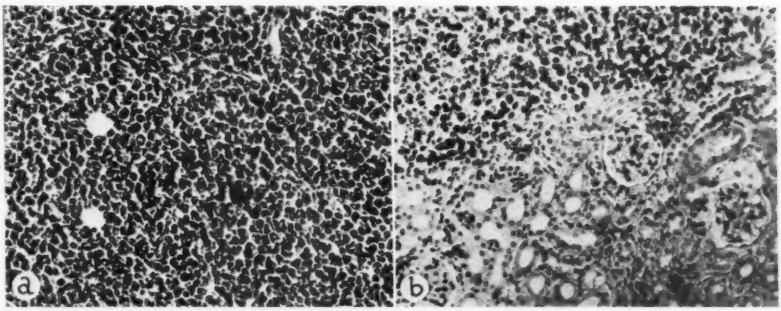

FIG. 1, a. Photomicrograph of tumor found in lymph glands of male rat No. 8.
b. Photomicrograph of tumor metastasis to kidney found in female rate No. 15.

the initial mating time and the delivery of the litters was prolonged in two instances, being 57 and 94 days. Rat No. 8 had now had 3 unfertile matings and at autopsy, on the 335th day of thiouracil administration, was found to have diffuse lymphosarcoma (Fig. 1, a) with markedly atrophied testes weighing 0.4 gm. in contrast to an average value of 1.8 gm. for the other treated animals. Rat No. 13 was obviously ill and died on the 280th day of treatment. As the animal was partially eaten no autopsy was possible. The remaining sterile mating occurred with rat No. 10 and is unexplained. The female partner was successfully remated. At autopsy all of the rats showed a marked increase in thyroid weight (Chart 2). Histologically the glands were hyperplastic (Fig. 2, a).

Thiouracil was next fed to a group of 6 month old female rats in

CHART 2. AUTOPSY DATA ON THIOURACIL FED MALE RATS

Days of drug administration	Body wt. gms.	Testes gms.	Adrenals mgms.	Pituitary mgms.	Thyroid mgms.	
60	282	2.4	—	—	39.8	
60	290	2.6	—	—	56.0	
	286	2.5	—	—	47.9	Ave.
—	295	2.7	—	—	18.5	Controls
211	301	1.9	25.0	18.5	111.0	
211	400	1.8	27.0	15.0	81.5	
211	357	1.8	26.0	19.0	112.0	
	352.6	1.8	26.0	17.8	101.5	Ave.
257	346	2.8	29.0	14.5	170.0	
257	405	3.6	38.0	19.0	113.5	
	375	3.2	33.6	16.7	141.7	Ave.
327	357	3.2	40.8	12.0	111.5	
327	395	3.5	20.0	17.0	161.5	
	376	3.4	30.4	14.5	136.5	Ave.
340	396	3.3	41.0	15.5	121.5	
340	304	3.0	30.5	11.0	159.0	
	350	3.1	35.7	13.2	140.0	Ave.
—	440	3.8	30.5	10.0	23.0	Controls

the same manner. Because of the difference in water consumption the calculated daily dosage was 20 mgm. per day of thiouracil per animal. Nine animals were mated 84 days after beginning thiouracil (Chart 3). Five delivered litters; 4 of these were normal and delivery occurred within 34 days after mating which is usual in our colony; the 5th rat

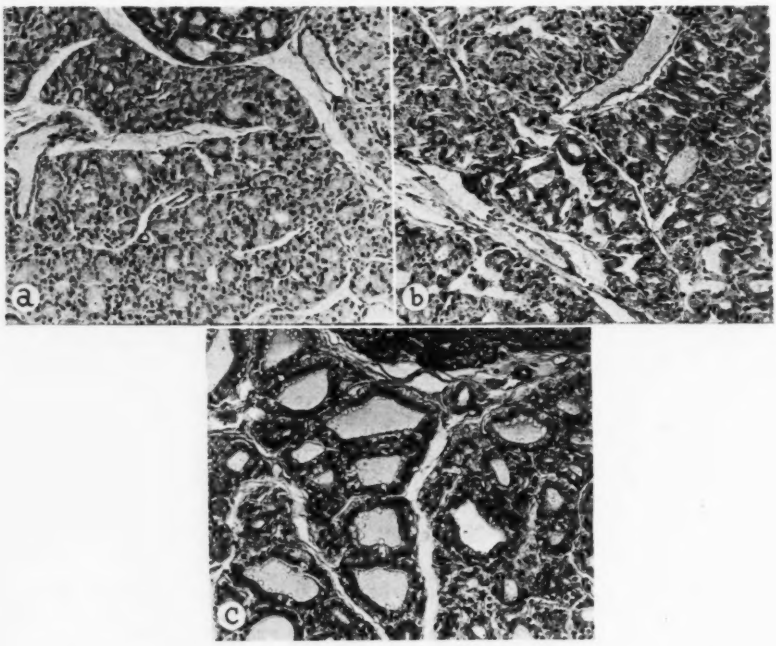

FIG. 2, a. Photomicrograph of thyroid of male rate after 340 days of thiouracil administration. There is extreme hyperplasia of glands with no colloid present. A portion of the parathyroid is also shown.

b. Photomicrograph of thyroid of female rate after 212 days of thiouracil administration. Although the hyperplasia is marked it is not as great as in Fig. 2, a. Some colloid can be seen in an occasional follicle.

c. Photomicrograph of thyroid gland from a control female rat showing dilatation of acini with colloid.

delivered only one pup and this 47 days after mating. One of the remaining 4 rats had normally palpable fetuses at 16 days after which they ceased to grow and bleeding several days thereafter indicated resorption. The other 3 rats had pregnancies which resorbed before the palpable stage.

Pregnancy and resorption have been judged by the occurrence of sperm in the smear, followed by diestrus smears and finally bleeding. Pseudopregnancy could give rise to this sequence of events, therefore 7 animals in the total series were laparotomized. Four had recogniza-

ble resorbing implantation sites and two had metrial glands depending upon the time relationship between the onset of bleeding and laparotomy. In addition to these 6 pregnancies proved by laparotomy,

CHART 3. RESULTS OF MATINGS OF FEMALE RATS FED A 0.1% SOLUTION OF THIOURACIL

Rat #	1st mating group			2nd mating group			3rd mating group			4th mating group		
	Days on drug	Day of del.	# in litter	Days on drug	Day of del.	# in litter	Days on drug	Day of del.	# in litter	Days on drug	Day of del.	# in litter
1	84	24	7									
2	84	29	5									
3	84	24	3									
4	84	34	8									
5	84	47	1									
6	84	—	resorp. d 16									
7	84	—	resorp.	134	—	resorp.	191	—	resorp. lap.			
8	84	—	resorp.	134	—	resorp.	191	—	resorp. lap.			
9	84	—	resorp.	134	—	resorp.	191	—	resorp. lap.	202	—	resorp. lap.
10				126	—	resorp.	191	—	resorp. lap.			
11				126	—	resorp.	191	—	resorp. lap.			
12				126	—	resorp.	171	—	resorp.			
13				126	—	resorp.	171	—	resorp.			
14				126	—	resorp.	171	—	resorp.			
15				126	—	resorp. lap.						

2 rats, Nos. 6 and 9, had pregnancies old enough to be palpable before resorbing.

The 5 rats in the first mating which delivered litters were excluded from the experiment as the thiouracil was discontinued during nursing. The pups were normal and reproduced normally.

The remaining females were mated, as indicated in Chart 3, 126,

CHART 4. AUTOPSY DATA ON TWO RATS WITH LYMPHOSARCOMA

Days of drug administration	Body wt. gms.	Gonad wt.	Adrenals mgms.	Pituitary mgms.	Thyroid mgms.
177	213	52.5 mgms. (tumor)	45.5	15.6	26.0
335	350	0.4 gms.	37.0	15.1	91.2

134, 171, 191 and 202 days after thiouracil administration. Of these 18 matings there were 18 resorptions, only one pregnancy progressing to the palpable stage. One rat, No. 15, was found to have lymphosarcoma (Fig. 1, b) at autopsy (Chart 4); her mating, however, had

resulted in a pregnancy as proved by laparotomy. The remaining 14 animals appeared to be in good health throughout the experiment. The thyroid glands were histologically hyperplastic (Fig. 2, b) and weighed an average of 36.5 mgm. in contrast to 21.1 mgm. for the control glands (Fig. 2, c). It is interesting to note that these glands did not increase as remarkably in size as did those of the male rats (Chart 5). None of the animals was autopsied before mating and each

CHART 5. AUTOPSY DATA ON THIOURACIL FED FEMALE RATS

Days of drug administration	Body wt. gms.	Ovarian wt. mgms.	Adrenals mgms.	Pituitary mgms.	Thyroid mgms.	
212	250	32.0	42.0	13.0	41.5	
212	232	40.0	45.0	18.0	33.0	
212	250	35.0	43.0	13.0	35.0	
	244	35.6	43.3	14.6	36.5	Ave.
—	272	61.7	51.6	14.6	21.1	Control

had had at least 2 pregnancies in fairly rapid succession. Perhaps the pregnancies interfered with the ability of the pituitary to produce thyrotropic hormone.

RESULTS AND DISCUSSION

Fourteen adult, thiouracil treated male rats were mated 22 times. There were 3 obviously ill animals with a total of 5 unsuccessful matings. If these animals are eliminated from the series there are 11 rats and 17 matings. Of these 11 rats 10 sired litters, Nos. 6 and 7 only one litter on repeated matings. Only one treated male (No. 10) therefore did not sire a litter and this one was mated only a single time with a single female. It seems probable that the tendency, seen in Chart 1, toward an increase of unsuccessful matings following the 166th day of thiouracil administration reflects the toxicity of the drug rather than any impairment in the reproductive capacities. The results indicate that hypothyroidism, as induced by thiouracil, has no effect upon the reproductive system of the male rat as judged by the ability to sire normal litters.

Fifteen female adult rats were mated at various intervals while receiving thiouracil. There were 27 matings, 22 of which resulted in resorptions. The 5 litters occurred in the first group of matings, made 84 days after the beginning of thiouracil administration; the remaining 4 matings of this group terminated in resorptions as did all 18 matings made after the 126th day of thiouracil administration. One interpretation of the findings may be that the matings of the first group reflect a state of borderline hypothyroidism, perhaps comparable to the period of about three months described by Engle which is necessary to dissipate the effect of thyroid feeding in previously thyroidecto-

mized monkeys (Ref. 2). Following the time lapse which may be necessary to produce severe hypothyroidism in rats, pregnancy does occur but terminates in resorption in 100 per cent of the cases.

The first question that arises is whether these results are due to some direct action of the thiouracil or to the hypothyroidism which it produces. If resorption in early pregnancy is a manifestation of thiouracil toxicity, it is an unusual one, for toxic drugs and illnesses usually produce sterility in rats rather than resorption of the embryos. It seems very unlikely that it is a direct effect upon the fetuses, since five matings of the first group resulted in normal litters, although the fetuses were exposed to the same amount of drug as were those in later experiments. The question can be answered directly by feeding thyroid to animals while under thiouracil treatment. Such studies are now in progress.

If the results do represent the effect of hypothyroidism on the reproductive system, they indicate that hypothyroidism has no effect upon gametogenesis in the rat but does have a marked effect upon the continuation of gestation producing resorption in mid or late pregnancy. How this effect is produced is a subject for further investigation though certain possibilities can be suggested. The hormones necessary for normal pregnancy in the rat are, as far as we know, pituitary- F.S.H., I.C.S.H. and luteotrophin, ovarian-estrogen and progesterone, and rat placental cyonin or chorionic gonadotropin. The pituitary F.S.H./I.C.S.H. ratio in thiouracil treated rats is apparently normal enough to produce ovulation and the luteotropin sufficient to maintain luteal function at a high enough level for implantation to occur. Estrogen and progesterone are being produced by the ovary in sufficient quantities for the maintenance of early pregnancy. If, then, the resorption of fetuses in these animals represents a hormone deficiency, as indeed it may not, it must be either a deficiency in cyonin of the placenta or an insufficient amount of estrogen and/or progesterone to maintain late pregnancy when the requirements for these hormones may be higher. In experiments on ovariectomized Rhesus monkeys Engle (Ref. 2) showed that thyroidectomy markedly raised the estrogen bleeding threshold while thyroid feeding restored it to normal. If it is true that thyroid controls the utilization of estrogen and perhaps progesterone our findings may be explained on this basis as normal amounts of estrogen and progesterone may have been rendered inadequate for the maintenance of late pregnancy by poor utilization of these hormones due to hypothyroidism.

SUMMARY

Hypothyroidism induced by thiouracil has no effect upon the reproductive system of the adult male rat as judged by the ability to sire litters. Prolonged thiouracil administration, associated with hypothyroidism, in the adult female rat, does not cause sterility but

does interfere with continuation of gestation, causing resorption of embryos in 100 per cent of the cases. If the drug is given over a period of less than 100 days some rats do deliver litters which are normal in growth and development and reproduce normally.

ACKNOWLEDGEMENT

We wish to thank Dr. R. O. Roblin of The American Cyanamide Company, Stamford, Connecticut, for supplying us with thiouracil.

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ACTIVATION OF THE ANTERIOR HYPOPHYSIS BY ELECTRICAL STIMULATION IN THE RABBIT[1]

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INTRODUCTION

BOTH the nature and extent of neural control over most of the endocrine organs are poorly understood, the exceptions including the posterior lobe of the hypophysis and the epinephrine-secreting chromaffin tissue. In the former group is one of the most obvious examples of an effect of nerve stimuli upon an endocrine organ, namely, the release of luteinizing hormone from the anterior lobe of the hypophysis in the rabbit and certain other forms upon sexual excitement.

The sources of sensory stimuli which activate the anterior lobe of the rabbit upon coitus have not been accurately determined. Fee and Parkes (1930) apparently first showed that ovulation in the rabbit does not depend purely upon genital stimulation, and similar results have been reported by others. As pointed out by Brooks (1937), it is possible that there may be many sensory and emotional pathways through which the ovulatory mechanism can be excited.

In spite of a number of careful investigations, the mechanism or mechanisms by which stimuli, of whatever source, act upon the anterior pituitary gland is also still subject to debate. Harris (1937) and Brooks (1938) felt that their experiments established the hypothesis that the nerve fibers in the hypophyseal stalk are essential to normal anterior lobe function in the rabbit. In contrast to earlier reports, recent evidence has shown that this is not true for either the rat or the guinea pig; in view of this work and especially that of Dey and his co-workers (1940, 1943a and b, etc.) on the hypothalamus of the guinea pig, the interpretation of the results of Harris and Brooks becomes much less convincing.

In the present experiments we have tried to secure, by means of electrical stimulation, further information on the relation of the nervous system to the anterior lobe of the hypophysis. Using the production of ovulation in the rabbit as the criterion for successful activation of the anterior pituitary gland, we have investigated the cervical

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sympathetic chain and the vagus nerves as possible routes to this gland. We have also stimulated the gland itself and the hypothalamus with currents of which the intensity and other electrical characteristics have been carefully recorded.

Our findings on the sympathetic and vagus nerves indicate that neither of these is part of a normal pathway involved in activation of the anterior pituitary gland; the results of stimulation of the hypophysis and of the hypothalamus suggest that the activation of the hypophysis by the hypothalamus is through mechanisms other than strictly neural ones.

MATERIAL AND METHODS

The rabbits used in these experiments were mature females; only those cases in which examination of the ovaries indicated such maturity are included in this report. The criterion of selection was the finding of ovaries of such size and condition that, on the basis of past experience, ovulation could be expected to follow injections of luteinizing hormone into the animal.

These animals, which had been isolated for a month previously, or whose ovaries had been examined at preliminary laparotomy, were electrically stimulated through (a) the cervical sympathetic chain, (b) the cervical vagus, (c) the hypophysis, and (d) the hypothalamus. A period of from 18 to 120 hours was allowed to elapse between the time of stimulation and the examination of the ovaries for freshly ruptured follicles. At autopsy, all of the ovaries were removed and examined under a binocular dissecting microscope; those in which there was any apparent indication of activation were serially sectioned for histological examination. In those cases in which the pituitary gland or the hypothalamus had been stimulated, the animal was perfused with saline and the sella, the hypophysis, and the hypothalamus were then examined to determine the position of the electrode.

The techniques of stimulation were purposefully varied in order to cover as wide a range of conditions as seemed practicable. For details of stimulation in specific cases, reference should be made to tables 2 and 3. The following is a general account of the methods used.

Stimulation of the cervical sympathetic chain in unanesthetized animals was effected by two different methods. In one series the cut upper end of the sympathetic chain was drawn through a glass tube in which it was held in contact with a bipolar electrode, leads from which were brought out through the skin. After a period of 2 hours in which the animal was allowed to recover from the effects of the ether used in the operation, stimulation was carried out for 10 minute periods, with intervening 2 minute rests, during the subsequent 2 hours. A Harvard inductorium with three volts attached to the primary was used as the source of stimulus, and so adjusted that while there was little evidence of spread, a marked dilation of the pupil was obtained.

In the second series of cervical sympathetic stimulations a buried electrode was also used, but in connection with a buried secondary coil. In these cases the electrodes were enclosed within a rubber sleeve which was fitted around the intact nerve, and the other ends of these electrodes were attached to a coil of 40 gauge insulated copper wire. This coil, containing 200 turns and about $\frac{3}{4}$ inch in diameter, was implanted beneath the skin and the wound was closed. After 1 to 10 days the unanesthetized animal was fas-

tened in a convenient position and the primary coil of a Harvard inductorium, connected to four dry cells, was brought near the previously buried secondary coil described above. The distance between primary and secondary coils was so adjusted that good pupillary dilation was obtained. The evidence of spread observed varied from minimal to marked contraction of the neck muscles.

Buried electrodes with attached leads brought out through the skin, as

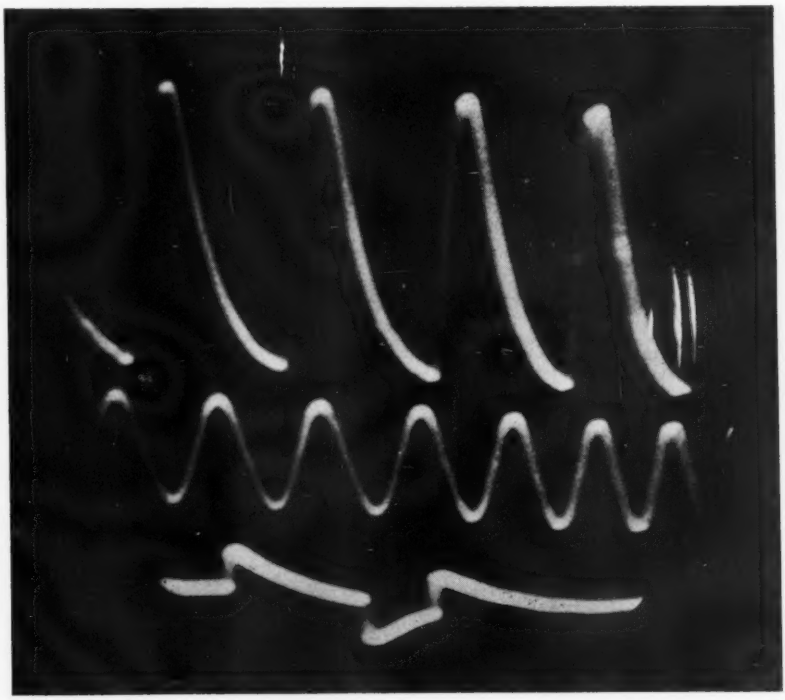

FIG. 1. An unretouched photograph of the cathode ray oscillograph screen showing, above, the voltage and the current delivered through the bipolar electrode at dial setting #4. In the middle is a 60 cycle time signal, and on the bottom the calibration (15 mv.).

in the first series of sympathetic stimulations, were also used to stimulate the vagus. In two cases, unipolar electrodes were used. Two to four days after these electrodes were implanted, stimulation was carried out over a total of from $1\frac{1}{2}$ to 3 hours, with 10 minute periods of stimulation alternating with 2 minute rest periods, or with 50-second periods of stimulation alternating with 10-second periods of rest. Various intensities of stimuli were tried in this series with from $1\frac{1}{2}$ to 6 volts attached to the primary of the Harvard inductorium and the secondary so placed that the discharge varied from one which produced no appreciable spread to one which produced very considerable spread to the forelimbs and neck. The inductoria delivered stimuli at

the rate of about 25 per second; in four cases a gear system was used to deliver shocks at the rate of 8 per second.

In direct stimulation of the hypophysis, and also in stimulating the hypothalamus, operations and stimulations were carried out under ether anesthesia. An electronic impulse generator, set to deliver about 40 impulses per second, was used as the source of the stimuli. The strengths of the stimuli delivered by bipolar and unipolar electrodes at certain of the dial settings indicated in tables 2 and 3 have been calibrated by meters for the average current of the stimulating impulse, and calculated for both current and voltage from photographs of the shape and height of the discharge on the screen of a cathode-ray oscillograph (Fig. 1). The average strengths of the stimuli for the critical dial settings #4 and #6, as measured in one animal, are given in table 1. Slight variations have been observed with shifts in the positions of the electrodes in the same animal, and upon repeated attempts to set the dial at the same point.

The anterior pituitary gland was stimulated directly with a bipolar electrode through the parapharyngeal approach, and with a unipolar electrode through both the parapharyngeal and temporal routes. The bipolar electrode was of the concentric type, with both electrodes insulated to the tips. The needle of this electrode was of 18 gauge; the central wire, which

TABLE 1. SHOWING THE AVERAGE MILLIVOLTS AND MILLIAMPERES DELIVERED BY THE IMPULSE GENERATOR AT DIAL SETTINGS #4 AND #6, USED IN STIMULATION OF THE HYPOPHYSIS AND OF THE HYPOTHALAMUS.

Dial setting	Type of stimulation	
	Bipolar	Unipolar
#4	110 mv. 0.037 ma.	135 mv. 0.039 ma.
#6	170 mv. 0.069 ma.	201 mv. 0.083 ma.

projected 1 mm. beyond the needle, was 22 gauge. The indifferent electrode used in unipolar stimulation was a copper plate of 7.5 sq. cm. which was usually fastened to the inner surface of the cheek with two bulldog clamps; the stimulating electrode was a 21 gauge wire. In nine animals in which unipolar stimulation was used, the intensity of the stimulus was kept constant; in seven of these, periods of stimulation alternated with shorter periods of rest. In the remaining cases, both of unipolar and bipolar stimulation, two strengths of stimuli were used in alternate periods of $2\frac{1}{2}$ minutes each, but there were no periods of rest.

Bipolar stimulation of the hypothalamus was carried out through the superior orbital fissure, but was otherwise similar to the bipolar stimulation of the anterior pituitary gland.

RESULTS

In the twenty-seven cases of stimulation of the cervical sympathetic trunk, twelve with electrodes attached to leads passing out through the skin and fifteen with buried secondary coils, examination of the ovaries revealed no rupture points and no freshly hemorrhagic

follicles. There was, therefore, no sign of activation of the anterior lobe of the hypophysis by the stimulations carried out in this series.

The vagus nerve was stimulated in twenty-two cases. In spite of the variations introduced into this series—prolonged stimulation time, changes in the number of stimuli per second, and variations in the strength of the stimulus from one producing practically no appreciable spread to one producing tetanic contraction of the neck and forelimb muscles—examination of the ovaries revealed negative results in every case.

Unipolar stimulation of the anterior lobe of the hypophysis (table 2) with a maximum dial setting of #4 or less (135 mv./0.039 ma.) was usually accompanied by little evidence of spread. This setting or a lower current and voltage were used exclusively in nine cases. In five of these the temporal approach was used and the electrode was introduced directly into the sella. In the other four cases the hypophysis was reached by the parapharyngeal approach; subsequent autopsy showed that the electrode had penetrated the anterior lobe in three cases, while in the fourth it had penetrated the sella but not the hypophysis itself. None of the ovaries of these nine animals showed a reaction which could be attributed to an activation of the anterior lobe of the pituitary gland.

Unipolar stimulation of the anterior lobe with dial settings reaching a maximum of #6 (201 mv./0.083 ma.) produced quite different results. The animals in this series were each stimulated at two intensities, these being alternated every $2\frac{1}{2}$ minutes. With the higher settings the most striking evidence of spread was marked depression of respiration, but in some cases there was also considerable spread to voluntary muscle. Of the six animals stimulated at these higher intensities, five had ovaries with visible rupture points on the follicles or fresh corpora lutea; the minimum was 2 and the maximum 6 in any one animal. In four of these cases, as in the one negative animal upon which this strength of stimulus was used, the electrodes were found upon autopsy to have been placed in the anterior lobe. The position of the electrode in the fifth successful case was not ascertained, as the animal was preserved for a further experiment.

Four animals were stimulated at an intermediate range of intensity, that is with a maximal dial setting of #5 (0.061 ma.). Of these, three had ovaries which gave no evidence of hypophyseal activation, although the electrode had been placed in the hypophysis in all of these cases. In the one positive case in this group, one ovary had 4 rupture points, the other one.

Bipolar stimulation of the pituitary gland at the same dial settings and with intensities of the same order of magnitude as those used in unipolar stimulation gave, in contrast to this latter, little evidence of spread. Respiration was not affected, and the spread seemed to be limited to a slight involvement of the eye muscles. The ovaries gave

TABLE 2. EFFECTS OF UNIPOLAR STIMULATION OF THE ANTERIOR LOBE

Wt. of animal kgms.	Stimulus			Amount of spread	Survival time, hrs.	Ovulation
	Total time	Alternations				
		Time	Intensity*			
2.5	60′	4½′ on ½′ off	2.4 Constant	Slight	21	−
2.25	15′	5′ on ½′ off	2.5 Constant	Slight	18	−
2.75	25′	5′ on 2′ off	2.8 Constant	Slight	20	−
2.25	30′	None	2.8 Constant	Slight	21	−
4.2	40′	4½′ on ½′ off	2.8 Constant	Slight	22	−
2.6	20′	4½′ on ½′ off	2.9 Constant	Slight	24	−
2.5	10′	None	3 Constant	Slight	20	−
2.6	40′	4½′ on ½′ off	3 Constant	Slight	22	−
2.9	45′	2½′ @ 2½′ @	2.8 4	Respiratory Inhibition	22	−
2.4	60′	4½′ on ½′ off	5 Constant	Respiratory Inhibition	40	−
2.7	40′	2′ @ 1′ @	3 5	Respiratory Inhibition	24	−
2.5	40′	2½′ @ 2½′ @	3 5	Much Spread	24	+
3.8	40′	2½′ @ 2½′ @	3 5	Respiratory Inhibition	21	−
2.25	40′	2½′ @ 2½′ @	4 6	Much Spread	22	+
4.1	45′	2½′ @ 2½′ @	3 6	Respiratory Inhibition	22	+
2.6	40′	2½′ @ 2½′ @	4 6	Respiratory Inhibition	46	+
2.25	40′	2½′ @ 2½′ @	4 6	Erratic Forced Abdominal Respiration	41	+
2.0	40′	2½′ @ 2½′ @	4 6	Respiratory Inhibition	18	−
3.2	40′	2½′ @ 2½′ @	4 6	Much Spread	24	+

* (In both tables 2 and 3.) The numerals refer to the dial settings. See table 1.

no evidence of pituitary activation in any of the seven animals in this series, which included one animal that had previously ovulated upon unipolar stimulation. In these seven the electrode had penetrated the anterior lobe in three and had penetrated the sella to rest against the surface of the hypophysis in the other four.

In the four cases in which the hypothalamus was stimulated by a bipolar electrode the stimulation was identical, in intensity and duration, with that used in the direct bipolar stimulation of the hypophysis which had yielded negative results. There was in these cases no effect upon respiration, but at the higher intensity a slight tremor appeared in the head, neck, and forelimbs. In three of these four cases

TABLE 3. EFFECTS OF BIPOLAR STIMULATION OF THE HYPOPHYSIS AND OF THE HYPOTHALAMUS

Wt. kgms.	Stimulus				Amount of spread	Survival time, hrs.	Ovulation
	Location	Total Time	Alternations				
			Time	Intensity*			
3.25	Hypophysis	40′	2½′ @ 2½′ @	4 6	None	24	–
3.3	Hypophysis	40′	as above	as above	Slight, to eyes	26+	–
3.3	Hypophysis	40′	as above	as above	Slight, to eyes	46	–
3.4	Hypophysis	40′	as above	as above	Slight, to eyes	46	–
3.0	Hypophysis	40′	as above	as above	Slight, to eyes	46	–
3.6	Hypophysis	40′	as above	as above	Slight, to eyes	20	–
3.4	Hypophysis	40′	as above	as above	Slight, to eyes	46	–
2.3	Hypothalamus	40′	2½′ @ 2½′ @	4 6	Tremor in head, neck, forelimbs	92	–
2.4	Hypothalamus	40′	as above	as above	Tremor in head, neck, forelimbs	92	+
2.7	Hypothalamus	40′	as above	as above	Tremor in head, neck, forelimbs	120	+
2.8	Hypothalamus	40′	as above	as above	Tremor in head, neck, forelimbs	120	+

examination of the ovaries indicated that the stimulation had induced a release of luteinizing hormone from the hypophysis. In one of these cases one ovary contained 6 rupture points and several hemorrhagic follicles, while the second ovary also presented several hemorrhagic follicles; in the second case each ovary had 2 rupture points with beginning luteinization of the follicle, and also hemorrhagic follicles which were luteinizing; in the third case there were 6 rupture points and 20 freshly hemorrhagic follicles; the fourth case showed no evidence of stimulation.

DISCUSSION

The results of this investigation agree with the basic conclusions of Harris (op. cit.), Haterius (1937), Brooks (1938), and others, that there is within the hypothalamus of the rabbit a neural mechanism

which can activate the anterior lobe of the hypophysis; activation of this mechanism in the female results in the release of luteinizing hormone and thus induces ovulation. In conjunction with other related work, however, the present results also seem to indicate that the activation of the hypophysis through this mechanism does not involve a direct nervous pathway which ends in the anterior lobe, but that the final link is probably a humoral one.

Possible motor pathways to the anterior lobe of the hypophysis have been discussed by Hinsey and Markee (1933), Harris, and others. The three main paths which have been suggested are the greater superficial petrosal nerve and the sphenopalatine ganglion; the cervical sympathetic chain; and the hypophyseal stalk. While Rosen, Shelesnyak and Zacharias (1940) and Zacharias (1942) have reported that removal of the sphenopalatine ganglion in the female rat induces pseudo-pregnancy, the hypophyseal effects of this operation are transitory except for an increased sensitivity to insulin. Vogt (1942) has shown that the great superficial petrosal nerve is not an essential pathway for excitation of the ovulatory mechanism in the rabbit, for females ovulated upon coitus after bilateral destruction of these nerves.

The cervical sympathetic trunk is also not essential to the ovulatory mechanism in the rabbit, for Vogt (1931, 1933), Hinsey and Markee (op. cit.), and Brooks (1938) have all reported that interruption of the cervical sympathetic chains does not prevent ovulation. However, as Friedgood and Pincus (1935) obtained ovulation or other evidence of hypophyseal-ovarian reaction following stimulation of the cervical sympathetic chains in the rabbit, the possibility that these nerves may afford a normal, even if relatively unimportant, pathway to the anterior lobe seemed worthy of reinvestigation. In the present experiments, all the animals in which the sympathetic was stimulated had recovered from the anesthesia and those with buried secondary coils had also ample time to recover from any untoward effects of the operation. In our hands, in the first series of sympathetic experiments, voltages which were apparently much less than those used by Friedgood and Pincus provoked such wide-spread contraction of voluntary muscle that they could not be regarded as delivering even approximately normal stimuli. In the present experiments, the higher voltages on the primary coil when used with a buried secondary were in part necessary to overcome skin resistance, and the current delivered to the nerve trunks was again so regulated that relatively little spread occurred. We have used the same make-break frequency as did Friedgood and Pincus; they report that this approximates the optimum for stimulation of sympathetic fibers. Since the twenty-seven cases of stimulation of the cervical sympathetic trunk, under conditions which we believe approach the optimum, have in our experiments failed to produce ovarian stimulation, we are inclined to

believe the cervical sympathetic chain plays no part normally in the activation of the hypophysis.

As Chang et al. (1937, 1938), Hsieh (1938), and Huang (1938) have reported reflex activation of the posterior lobe of the hypophysis from stimulation of the central end of the cut vagus, it seemed possible that the positive results of Friedgood and Pincus might have been due to stimulation of sensory fibers of this nerve. Such stimulation could be due to direct spread or to the presence within the sympathetic trunk of vagus fibers; in the cat (Hollinshead, 1939, 1940) admixtures of the two sets of fibers are apparently fairly common. However, our twenty-two cases of vagal stimulation were all negative, although various strengths of current were used, and make-break frequencies similar to that used by Friedgood and Pincus on the sympathetic, and that used by the Chinese workers on the vagus, were both employed. From these present experiments, therefore, we have no evidence that either cervical sympathetic or vagal activity will affect the activity of the anterior hypophysis. Since Marshall and Verney (1936) using rabbits, Harris (1936) with the rat, and Ellis and Wiersma (1945) in the guinea pig and the dog, have all demonstrated that very strong electrical stimuli which are not localizable will influence hypophyseal activity, it seems possible that the difference between the positive results of Friedgood and Pincus and our own negative results may be due to differences in the voltages employed, and differences in the amount or direction of spread.

While a relation between the hypothalamus and the activity of the anterior lobe of the pituitary gland, as reflected in gonadal functions, has long been indicated, the relation of the hypophyseal stalk to this activity has been less clearly demonstrated. As long ago as the work of Camus and Roussy (1920) it was shown that damage to the hypothalamus might lead to genital atrophy. Dey and his coworkers (1940, 1941, 1942, 1943 a and b) have analyzed the effects on the hypophysis of variously-placed lesions in the hypothalamus of the guinea pig and report that lesions of the median eminence lead to genital atrophy, while lesions of the anterior hypothalamus and supraoptic nuclei produce in the female a state of constant estrus. Haterius (op. cit.) has produced ovulation in the rabbit by stimulating the hypothalamus with a stereotactically controlled bipolar electrode, and Harris (1937) has also obtained this result by unipolar stimulation of the hypothalamus. Both investigators express the opinion that the electrical stimuli used did not spread to the pituitary gland itself, and that they were therefore activating a neural mechanism within the hypothalamus.

In these present experiments, a similar result has been obtained. Using bipolar stimulation to the hypothalamus at voltages and amperages only a fraction as great as those used by Harris, we have obtained ovulation in three out of four experiments. Moreover, we

have shown that a similar stimulation of the pituitary gland itself, under otherwise exactly the same conditions, has not provoked pituitary activity. There can thus be little question that the present results are due to hypothalamic stimulation per se, rather than a spread of the electric current to the hypophysis. Our experiments offer no data upon the exact localization within the anterior hypothalamus of the neural mechanisms so stimulated, but do bear evidence to the relative ease with which they can be excited by electrical means.

The importance of the hypophyseal stalk as a direct nervous connection between the hypothalamus and the posterior lobe of the hypophysis has been beautifully demonstrated by Fisher and his coworkers (1938). It is generally reported that the distribution of nerve fibers within the anterior lobe is quite sparse, but Brooks and Gersh (1941) have described the passage of fibers from the stalk and posterior lobe into the anterior lobe in both the rat and rabbit; they believe that in this latter animal the fibers so described constitute the pathway through which the anterior lobe is activated to release luteinizing hormone. Truscott (1944) has recently studied in more detail the passage of hypothalamic fibers into the anterior lobe of the hypophysis of the rat, and has reported a surprising number of such fibers.

While there is thus anatomical evidence of the existence of a nerve pathway between the hypothalamus and the anterior lobe of the hypophysis, the physiological importance of this pathway is debatable. Although Richter (1933) reported prolongations of the estrus cycles of rats following section of the stalk, Dempsey and Searles (1943) have found that section of the stalk did not necessarily interfere with the estrus cycle in their rats. Further, they have demonstrated that the effects of light, heat, and cold upon the estrus cycles of rats are still produced in animals with sectioned pituitary stalks. Dey (op. cit.) similarly agrees that section of the stalk in the guinea pig does not disturb the estrus cycle, although hypothalamic lesions do.

Working on the rabbit, Harris (1937) has reported that lesions of the stalk in this animal lead to degeneration of the cells of the anterior hypophysis and consequent atrophy of the sexual organs. Brooks (1938) found that section of the hypophyseal stalk in his animals prevented the ovulation normally induced by coitus, and Brooks, Beadenkopf and Bojar (1940) have reported that this operation will also prevent the ovulation which can in normal animals be induced by the injection of copper acetate and certain convulsive drugs. Since the rabbit, unlike the guinea pig and rat, normally ovulates only following sexual excitement, it may of course possess a specific hypothalamico-hypophyseal mechanism which is entirely lacking in these other rodents. However, in view of the more recent reports of the results of hypophyseal stalk section in these other forms, the

validity of the conclusions reached from the above findings on the rabbit is obviously open to serious doubt. To what extent these findings may be due to interruption of the nerve pathway or to coincidental hypothalamic injury, and to what extent, if any, they are due to damage to the hypophyseo-portal system, is not clear.

While our own experiments have not involved section or stimulation of the hypophyseal stalk, we believe they do bear upon the possibility of a direct nervous pathway from the hypothalamus to the anterior lobe of the hypophysis. Examination of table 2 will show that in our experiments unipolar stimulation of the hypophysis never produced ovulation at strengths below and up to 135 mv./0.039 ma.; stimulation with voltages and currents between those figures and 201 mv./0.083 ma. produced ovulation in one case out of four, and stimulation with this latter intensity produced ovulation in five out of six animals. With the higher voltages used in unipolar stimulation there was obvious spread to other parts of the nervous system, and only when such spread existed was ovulation obtained from direct pituitary stimulation. It seems probable, therefore, that our positive results in this series are due not to activation of the pituitary gland directly, but to the spread of the electrical excitation to the hypothalamus and activation of the centers there.

This conclusion is borne out by the results of bipolar stimulation (table 3) from which it will be seen that identical stimulation of the hypophysis and hypothalamus gave consistently negative results in the former and 75% positives in the latter. How much the bipolar stimulation used here was above the threshold of stimulation of the hypothalamus is not known, for only this particular voltage was used upon the hypothalamus. Without regard to this latter point, however, it is clear that the hypothalamic mechanism is much more easily excited electrically than is the hypophysis itself. Although Harris (1937) reported the threshold of unipolar stimulation was the same for both pituitary gland and hypothalamus, our results indicate that for the latter, at least, he far exceeded the threshold.

If one assumes that the results of the hypothalamic stimulation reported here are due to transmission of impulses along a nerve pathway which ends in the anterior lobe of the pituitary gland, the differential excitability of these fibers in the hypothalamus, on the one hand, and the gland itself on the other, offers decided difficulties of interpretation. Taubenhaus and Soskin (1941) have obtained results indicating that a neurohumoral agent may be concerned in stimulation of the anterior hypophysis in the rat, and our own results are most logically interpreted on the basis that there is a humoral link between hypothalamus and anterior lobe of the hypophysis. While the results of Taubenhaus and Soskin might indicate that cholinergic nerve fibers end within the anterior hypophysis, the present results

indicate rather that there is no direct nervous pathway, and that probably, therefore, a long humoral link forms a part of the hypothalamico-anterior hypophyseal connection.

SUMMARY

Stimulation of the cervical sympathetic trunk in twenty-seven unanesthetized mature female rabbits has given no evidence that the release of luteinizing hormone from the anterior lobe of the hypophysis can be induced by normal activity of these nerves. The possibility that sensory fibers of the vagus might reflexly affect the activity of the anterior lobe, as they are said to do for the posterior, is also apparently negated by a series of twenty-two cervical vagus stimulations, following none of which were ovarian changes observed.

Direct unipolar stimulation of the anterior pituitary gland in nineteen animals, with voltage and current up to an average maximum of 201 mv./0.083 ma. induced ovulation only in those animals in which evidence of considerable spread to the central nervous system was observed. Bipolar stimulation of the pituitary gland, with average voltage and current of the same order of magnitude as those used in unipolar stimulation, was accompanied by no evidence of spread to the central nervous system, and failed to effect ovulation in all seven cases. Bipolar stimulation of the hypothalamus, however, under the identical conditions found ineffective on the hypophysis, induced ovulation in three out of four animals.

The above experiments confirm the findings of other workers that the hypothalamus plays a major role in the release of luteinizing hormone from the anterior lobe of the hypophysis. However, the difference in the electrical excitability of the hypophysis and of the hypothalamus, as shown here, is interpreted as indicating that the hypothalamus does not exert a direct neural control over the anterior hypophysis; rather, the presence of a long humoral link seems to be implied by the results of these experiments.

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ADENOSINE TRIPHOSPHATASE ACTIVITY OF LUTEIN AND OVARIAN TISSUES AND WEIGHT OF CORPORA LUTEA DURING THE REPRODUCTIVE CYCLE OF THE RAT[1]

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IT IS WELL KNOWN that the adenosine triphosphate system occupies a key position in the energy exchanges of muscle during glycolysis. The presence of ATP and the ATP-splitting enzyme, ATP-ase, in variable quantities in a number of additional tissues has been reported (DuBois and Potter, 1943), and evidence has been presented pointing to the ATP system as a unique mechanism in linking the energy-requiring reactions of tissues with the energy-yielding reactions (Potter, 1945). These reports suggested that the energy changes occurring in tissues other than muscle might be mediated through the ATP system. We became interested, therefore, in determining whether or not the ATP-ase activity of ovarian tissues varied during the different phases of the reproductive cycle, as it is well known that these tissues undergo rapid changes in function and structure at these times. The studies which are reported here were made on the ATP-ase activity of lutein and remaining ovarian tissues during the reproductive cycle. A portion of these results have appeared in preliminary form elsewhere (Biddulph, Meyer and McShan, 1946).

MATERIALS AND METHODS

Virgin female rats of the Sprague-Dawley strain 3½ to 4½ months old were used. Vaginal smears were made on all of the animals to establish normal estrous cycles, after which ATP-ase determinations were made upon one group in late estrus and another group on the second day of diestrus. A third group was made pseudopregnant by means of cervical stimulation and ATP-ase determinations were made at 4, 7, and 11 days of pseudopregnancy. A fourth group was placed with males and allowed to become pregnant, and ATP-ase determinations were made at intervals throughout pregnancy and lactation.

Pregnancies were dated from the time sperm were found in the vaginal smear. Following insemination the females were caged individually and were later sacrificed by decapitation at 4, 7, 11, 15 and 20 days of gestation. At

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autopsy the ovaries were removed and placed on moist, hard-surfaced filter paper and the corpora lutea were dissected free with sharp-pointed scalpel blades under spectacle loupes. The tissue was immediately weighed and placed in a 15 ml. tapered homogenizing tube containing 0.1 ml. of glass distilled water which was kept in a beaker of ice. The ovarian residue was dissected free of all remaining discernible corpora lutea (if present) and likewise weighed and placed in a chilled homogenizing tube.

For the lactation studies all litters were uniformly reduced to 6 young on the day of birth in an effort to standardize as much as possible the lactation rate of the mothers. These rats were likewise sacrificed on days 4, 7, 11, 15 and 20 of lactation. In a number of instances the young were removed from the mothers on day 20, and daily vaginal smears were begun on the latter for the purpose of determining the time of reappearance of estrus. Animals of this type were sacrificed on days 22 and 23.

The ovaries of lactating rats contain two sets of corpora lutea, the corpora of pregnancy and the corpora of the post-partum ovulation (Long and Evans, 1922). With a little practice it was possible to rapidly and accurately identify each type of corpus luteum. The identification was confirmed in a separate study by placing fine strands of surgical silk through the base and through the center of individual corpora of pregnancy early in lactation with the finest obtainable embroidery needle, and then autopsying and examining the ovaries at intervals thereafter. The dissection of the two sets of corpora lutea and their subsequent treatment, as well as that of the ovarian residue, was the same as that described above.

The tapered, thick-walled homogenizing tubes were fitted individually to tapered glass rods by grinding with a little carborundum and water. With properly fitted tubes, an extremely fine homogenate was prepared. After the tissue samples were homogenized, sufficient cold glass distilled water was added to give a 1 per cent homogenate.

Determination of the ATP-ase activity of the homogenized tissues was made using essentially the method of DuBois and Potter (1943). The following modifications were made from their method: 1) 0.20 ml. of 0.013 M ATP was used instead of 0.15 ml., 2) 0.2 ml. of 50 per cent trichloracetic acid was used to stop the incubation reaction and to precipitate the proteins instead of 0.1 ml., and 3) 0.3 ml. of the supernatant from the digest in a final volume of 10.0 ml. was employed in the Evelyn colorimeter in determining the inorganic phosphorus (Fiske and Subbarow, 1925).

The ATP-ase activity of each tissue sample was determined throughout at three different tissue levels, namely, 1.0, 1.5 and 2.0 mg. of fresh tissue. This served to check the validity of the results by assuring that the activity of the enzyme was always proportional to the amount of tissue reacting. Three animals were used on each point, so each value in the tables and figures is an average of 9 determinations. The results are expressed as units of ATP-ase activity per mg. of fresh tissue, a unit being defined as the quantity of ATP-ase which will liberate 1 μg. of inorganic phosphorus from ATP in 15 minutes at 37° C. (DuBois and Potter, 1943).

The ATP used as substrate was prepared from rabbit muscle by the method of Needham (1942). This preparation was subjected to 7 minute hydrolysis in normal HCl and the phosphorus liberated was determined. The results show that the preparation was 80 per cent pure.

RESULTS

The curves in figure 1 are typical of the results of an ATP-ase determination on the 3 tissue levels used. These demonstrate that the enzyme activity was proportional to the amount of tissue reacting. The lower curves show the effect of adding and withholding calcium from the incubation mixture when the same sample of lutein tissue was used. Calcium has been shown to activate the ATP-ase of skeletal muscle (Bailey, 1942), and of other tissues (DuBois and Potter, 1943). Furthermore, ATP is the only compound whose dephosphorylation is significantly activated by calcium (DuBois and Potter,

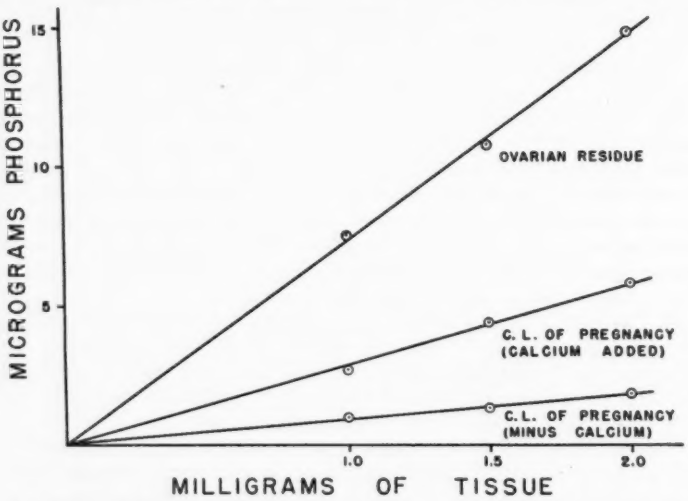

FIG. 1. ATP-ase activity of ovarian and lutein tissues as related to the amount of tissue reacting, and the effect of calcium on the ATP-ase activity of lutein tissue.

1943). Calcium was added to the incubation mixture in all determinations of the present study.

Estrous Cycle: The ATP-ase activity of newly formed corpora lutea removed in late estrus approximately 12 hours after their formation, of corpora lutea on the second day of diestrus, and of the ovarian residue during estrus and diestrus, is presented in table 1. It will be noted that the ATP-ase activity of the corpora lutea was slightly greater in diestrus than in estrus, even though the weight of the corpora in each phase was about the same. Although the difference in the values of the ovarian residue between the estrus and diestrus phases is small, it is noteworthy that the lowest ATP-ase activity was found at a time when the estrogen level in the ovaries was the highest, and conversely that the highest ATP-ase activity was found when the estrogen level was lowest. It should be pointed out that the ovarian

residue is composed chiefly of interstitial, germinal and stromal tissues, as great care was taken to dissect out all discernible corpora lutea. Consequently the ovarian residue used in these studies undoubtedly contained the chief estrogen-producing elements of the ovary.

Pseudopregnancy: The data in table 1 also show the ATP-ase ac-

TABLE 1. ADENOSINE TRIPHOSPHATASE ACTIVITY OF LUTEIN TISSUE AND OVARIAN RESIDUE AND WEIGHT OF THE CORPUS LUTEUM DURING THE REPRODUCTIVE CYCLE.

Stage	Corpora lutea		Ovarian residue
	Average number units[1] ATP-ase	Average weight per corpus luteum (mg.)	Average number units[1] ATP-ase
Estrus	7.8±0.47[2]	1.2	13.6±0.54
Diestrus	9.8±0.60	1.1	15.7±0.27
Pseudopregnancy:			
4 days	7.2±0.32	1.5	18.0±0.25
7 days	7.9±0.54	1.6	17.6±0.84
11 days	8.1±0.40	—	11.2±0.57
Pregnancy:			
4 days	7.4±0.15	1.5	13.5±0.74
7 days	7.8±0.31	1.4	19.7±0.66
11 days	7.5±0.17	2.1	13.8±0.22
15 days	5.8±0.81	4.0	14.3±0.82
20 days	9.2±0.17	4.5	12.6±0.20
Lactation:			
4 days	15.9±0.13[3]	2.4	16.3±0.64
	5.5±0.24	1.5	
7 days	16.6±0.76	1.6	17.7±0.43
	7.5±0.65	1.8	
11 days	24.9±0.95	0.7	19.8±1.30
	7.8±0.71	1.5	
15 days	20.6±0.43	0.6	16.8±0.59
	6.7±0.28	1.7	
20 days	16.5±0.87	0.5	15.0±0.23
	9.1±0.47	2.1	
22 days	16.4±0.88	0.4	16.0±0.78
	12.3±0.57	2.2	
23 days	15.4±1.30	0.5	14.7±0.83
	9.8±0.40	2.9	

[1] A unit is the quantity of ATP-ase which liberates 1 μg. of inorganic phosphorus from ATP in 15 minutes at 37° C. The values are expressed as units per mg. of fresh tissue, each value being the average of 9 determinations (3 for each of 3 rats).

[2] Standard error.

[3] First line, corpora of pregnancy; second line, corpora of the post-partum ovulation.

tivity of the corpora lutea and ovarian residue during pseudopregnancy. It will be seen that the activity of the corpora lutea did not change markedly from the levels found during the estrous cycle. Neither was there any marked change in the enzyme activity of the corpora lutea from the earlier to the later stages of pseudopregnancy. The enzyme activity of the ovarian residue, however, decreased greatly on the 11th day as compared to days 4 and 7. As has been suggested for the ovarian residue during estrus, the lower enzyme level

of the ovarian residue at the end of pseudopregnancy is likewise inversely related to the estrogen level of the ovarian tissue, for a high percentage of rats of this strain show the reappearance of estrus around the 11th to 12th days of pseudopregnancy. The uteri of the animals used on the 11th day of pseudopregnancy were hyperemic and contained fluid, which are evidences of estrogen production, and the ovarian residue also contained a few medium to large follicles, which indicates that the estrogen level was higher at this time than during the earlier stages of pseudopregnancy.

Pregnancy and Lactation: The results of the enzyme determinations and the weight changes of the corpora lutea during pregnancy and

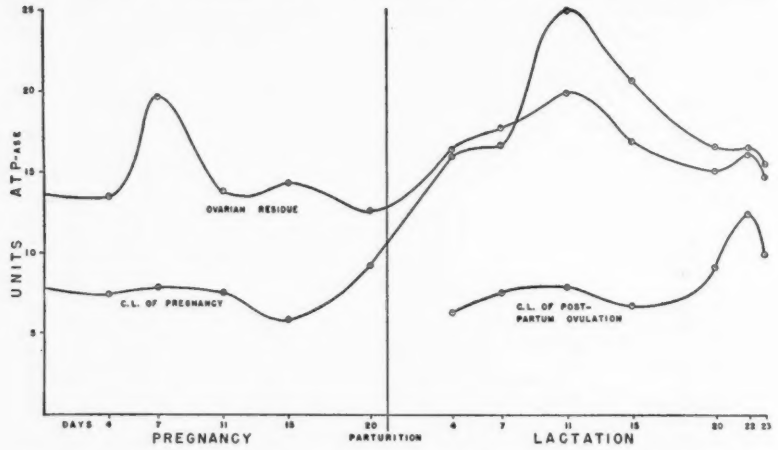

FIG. 2. ATP-ase activity of corpora lutea of pregnancy, corpora lutea of the postpartum ovulation, and the ovarian residue during pregnancy and lactation.

lactation are presented in table 1 and figures 2 and 3. The enzyme activity of the corpora lutea during the first 11 days of pregnancy was practically identical with that found in newly formed corpora and during pseudopregnancy. On day 15 a slight reduction in the ATP-ase occurred, which was followed by a rise shortly before parturition that continued until the 11th day of lactation. After day 11 the enzyme activity slowly decreased until day 23 of lactation, at which time the level was still considerably above that found during any part of pregnancy.

The corpora lutea did not show a significant increase in weight until the 11th day of gestation. On day 15 the increase continued and by day 20 the maximum average weight of the corpora was attained. The weights of the corpora lutea during pregnancy reported by Laqueur (1945) are almost identical with those found in our study. Following parturition the average weight of the corpora of pregnancy

decreased very rapidly until day 11, after which only a slight decrease occurred.

During gestation the ATP-ase activity of the ovarian residue increased markedly between days 4 and 7, and decreased greatly between days 7 and 11, reaching its lowest value on day 20. The activity again rose on day 4 of lactation, reaching its maximum on day 11. This was followed by a decline until the end of the experiment.

The variations in ATP-ase activity of the corpora of the post-partum ovulation during the first 20 days of lactation are remarkably similar to those of the corpora of pregnancy during gestation. A further correlation is the increase in activity that occurred shortly after

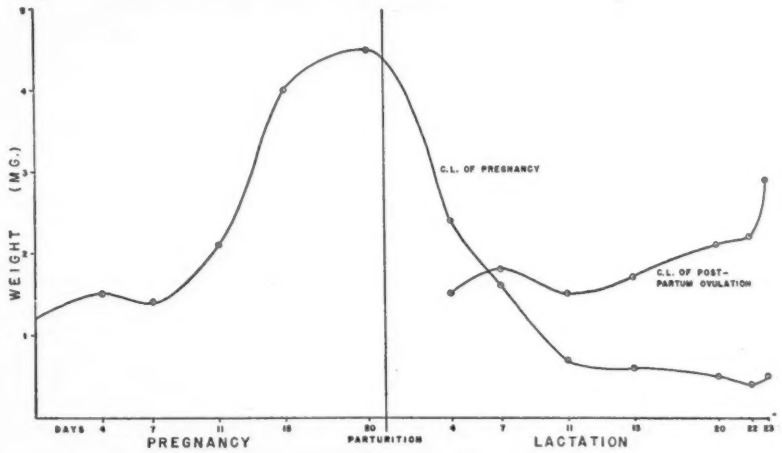

FIG. 3. Average weight of the corpora lutea of pregnancy and corpora lutea of the post-partum ovulation during pregnancy and lactation.

day 20 in both sets of corpora lutea. The increase in ATP-ase activity after day 20 of lactation was correlated with the cessation of suckling by the young and the beginning of estrous cycles. As mentioned above, the young were removed and daily vaginal smears begun on day 20. One of the rats used on day 22 showed an early estrus vaginal smear, and one of the rats used on day 23 showed a full estrus smear with follicles in the ovaries at the time of autopsy. It seems probable that estrus was imminent in the remaining animals at these times.

The average weight of the corpora lutea of the post-partum ovulation progressively increased throughout the period of lactation except for a decline on day 11. Following the cessation of suckling on day 20, a maximum value was reached on day 23. It would be interesting to follow, if possible, both the ATP-ase activity and weight of this set of corpora lutea for a longer period of time so that further comparisons could be made with the corpora of pregnancy.

DISCUSSION

The ATP-ase values of the tissues as they have been presented above are in terms of activity per milligram of fresh tissue. It is obvious, however, from inspection of the data in table 1 that during pregnancy the ATP-ase activity of the corpora of pregnancy remained fairly constant during the time that the corpora lutea were rapidly increasing in absolute size, and that following parturition the ATP-ase activity increased rapidly during the period that the corpora lutea were rapidly decreasing in size. The presentation of the data in such a manner does not accurately convey the changes that are taking

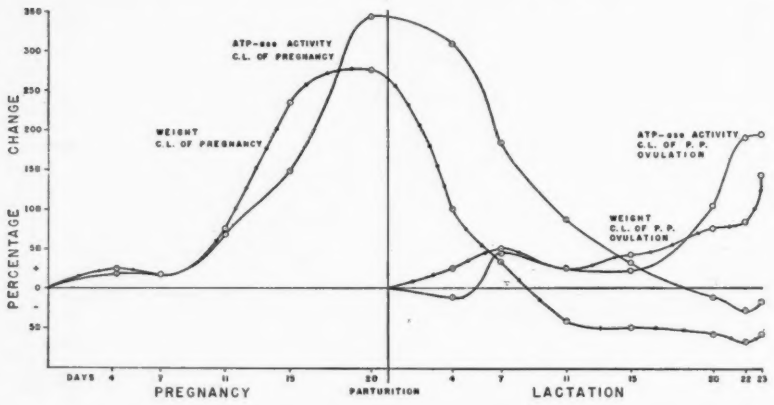

FIG. 4. Percentage change from the late estrus level in ATP-ase activity and weight of the corpus luteum during pregnancy and lactation.

place in a single corpus luteum. The changes in ATP-ase activity per corpus luteum would seem to be a better criterion for correlating the ATP-ase activity with the function of the corpus luteum. Therefore, the average total ATP-ase activity of the corpora of late estrus, of pregnancy, and of the post-partum ovulation was calculated. Further, the percentage change in ATP-ase activity and in weight of the corpora of pregnancy during pregnancy and lactation and of the corpora of the post-partum ovulation was calculated using the ATP-ase activity and weight of the corpora of late estrus as a base. The graphs of these changes are presented in figure 4 and these show that the weight and the ATP-ase activity of the corpora of pregnancy increased at identical rates during the first 11 days of gestation. On day 15 the increase in ATP-ase activity was not quite as rapid as the increase in weight, but before day 20 the relationship had changed so that at that time the increase in ATP-ase activity was 67 per cent greater than the increase in weight. After parturition both curves declined at approximately the same rate, but the absolute change in

percentage ATP-ase activity was always greater than the absolute change in percentage weight.

In general, the same relationship between the percentage change in ATP-ase activity and weight of the corpus luteum holds for the corpora of the post-partum ovulation during 20 days of lactation. With the cessation of suckling the ATP-ase activity increased more rapidly than the weight, whereas during lactation the percentage change in ATP-ase activity was either equal to or slightly less than the percentage change in weight.

It seems probable, as suggested by Laqueur (1945), that the increase in size of the corpus luteum during pregnancy is due to enlargement of the individual lutein cells rather than to the formation of additional ones. The above results indicate, therefore, that while the corpus luteum is growing in size during pregnancy the relative ATP-ase activity per lutein cell remains approximately the same and likewise during lactation as the lutein cell decreases in size, the relative activity per lutein cell remains approximately the same. The absolute ATP-ase activity per lutein cell of the corpora of pregnancy, however, during the first 11 days of lactation is greater than that during the growth phase of pregnancy. The absolute ATP-ase activity per corpus luteum decreases faster than the weight from the 11th to the 23rd day of lactation, due perhaps to degeneration and death of the cells of the corpora of pregnancy.

Progesterone output by the functional corpus luteum is very marked during the first half of pregnancy, as determined by its action on the endometrium of the mouse (Atkinson and Hooker, 1945). During this time there is relatively a smaller quantity of ketosteroids (principally progesterone) contained within the corpus luteum than later in pregnancy, which also indicates that progesterone is being liberated from the lutein cell at a faster rate early in pregnancy than in the later stages (Laqueur, 1945). Dempsey and Bassett (1943) have likewise shown by histochemical methods that the amount of steroid contained in the corpus luteum is least during the period of greatest secretory activity. The results of the present study show that the ATP-ase activity was relatively lower in the corpus luteum during the first half of pregnancy when the output of progesterone was great, and relatively higher just before parturition and thereafter when the output of progesterone (Atkinson and Hooker, 1945), but not the amount in the corpora (Laqueur, 1945), was relatively low. During the period of high ATP-ase activity of the corpora lutea ketosteroids (principally progesterone), cholesterol and non-steroid lipids also accumulated within the corpora lutea (Laqueur, 1945).

From the evidence just cited, indicating that progesterone secretion occurs at a time when the ATP-ase activity was relatively low, it would seem that during the period of active milk production by the mammary glands the corpora lutea of the post-partum ovulation are

actively secreting progesterone, as the ATP-ase activity is low at this time. With the termination of lactation, progesterone or its lipid precursors begin to accumulate in the corpus luteum, as does the ATP-ase activity, and shortly thereafter regression of the corpora begins. That progesterone output is high during most of lactation has recently been confirmed by additional studies reported by Atkinson (1946).

Lardy, Hansen and Phillips (1945) and Lehninger (1945) have reported that the ATP system is involved in lipid metabolism. These results and the correlation of the present results with those of Laqueur and others cited above, provide the basis for suggesting that the ATP system may be concerned with the lipid metabolism of the corpus luteum, possibly with progesterone formation and release.

The level of estrogen throughout pseudopregnancy, pregnancy and lactation in the mouse has been determined by Atkinson and Hooker (1945) and Atkinson (1946). Comparison of the ATP-ase activity of the ovarian residue with the data of these workers reveals a striking correlation. On days 4 and 7 of pseudopregnancy the estrogen effect was very low, but by day 11 it had increased to a level above that found on day 1. Our data show a high ATP-ase activity on days 4 and 7 of pseudopregnancy and a low activity on day 11. At the latter time estrous cycles were beginning to reappear in our animals. During pregnancy the estrogen effect was low on days 4 and 7 but increased somewhat after day 10, reaching a maximum on day 20. Within 2 days after parturition the evidence of estrogen activity disappeared. A marked increase in ATP-ase activity was found on day 7 of gestation when the estrogen effect was lowest, followed by a decrease on days 11 and 15 when the estrogen effect was increasing. The lowest ATP-ase value was obtained on day 20 when the estrogen effect was highest. Then followed an increase in ATP-ase on days 4, 7 and 11 of lactation, while the estrogen effect disappeared by the second day of lactation. Thus an inverse relationship holds for every stage of pseudopregnancy, pregnancy and lactation at which direct comparisons are possible.

SUMMARY

Determinations of ATP-ase activity and weight of lutein tissue and of the remaining ovarian tissue were made during estrus, diestrus, pseudopregnancy, pregnancy and lactation in the rat.

In general, the ATP-ase activity was lower in functional corpora lutea than in apparently non-functional corpora when the results were considered on the basis of per unit weight of tissue. When considered on a per corpus luteum basis, the weight and enzyme activity increased and decreased at approximately the same rate during pregnancy and lactation.

The enzyme activity of the ovarian tissue remaining after removal of the corpora lutea was greatest on the 4th day of pseudo-pregnancy, 7th day of pregnancy and 11th day of lactation.

The corpus luteum increased markedly in weight during the last half of gestation, reaching its maximum weight on day 20. The weight of the corpus luteum of the post-partum ovulation increased as lactation declined or ceased entirely.

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LOCAL INHIBITION OF HAIR GROWTH IN DOGS BY PERCUTANEOUS APPLICATION OF ESTRONE[1]

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THE SEX DIFFERENCES in the structure and appearance of portions of the integument and its appendages of man and of some other animals are often striking and generally recognized. Frequent investigations have been undertaken to determine the extent to which these variations in the dermis, epidermis, hair follicles and cutaneous glands are determined by endocrine factors. The diagnosis of certain endocrine disturbances may be aided by study of the condition of the skin and distribution of hair.

Partial alopecia occurs in dogs exhibiting symptoms associated with the excessive production of intrinsic estrogens (Greulich and Burford 1936; DeVita 1939). The subcutaneous injection of estrogens in large amounts almost completely stops the growth of hair in dogs (Gardner and DeVita 1940). Thinning of the hair occurred in dogs following the oral administration of stilbestrol (Mulligan 1944). In rats hair growth was inhibited when estrogenic hormones were administered in amounts adequate to inhibit general somatic growth (Zondek 1937; Hooker and Pfeiffer 1943; Emmens 1942).

How estrogen alters the growth of hair is unknown. Hooker and Pfeiffer (1943) assumed that the detoxification of estrogens and the increased epithelial proliferation, such as in the vagina, might reduce the available cystine necessary for the growth of hair. Estrogen-treated rats fed diets supplemented by casein failed to show a more rapid growth of hair than the estrogen-treated controls. This indicated that the hypotrichosis was not due to the ingestion of insufficient amounts of cystine.

Estrogens have elicited some reactions indirectly by first stimulating the pituitary gland which in turn acted upon an end organ. The local action of estrogen on the hyperemia and pigmentation of the areolar and sexual skin and of the mammary glands has been determined by direct application in man (MacBryde 1939), monkeys (Speert 1940; Chamberlin, Gardner and Allen 1941), rabbits and mice

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(Lyons and Sako 1940). Cutaneous application of very small amounts of estrogens induced hyperaemia of the treated sexual skin in monkeys while the rest of the sexual skin was not altered (Chamberlin, Gardner and Allen 1941)

The direct action of estrogens on the skin of dogs was investigated by applying percutaneously these chemicals in amounts insufficient to induce systemic reactions.

MATERIALS AND PROCEDURES

Three pure bred female toy fox terriors ranging in age from 2 months to 2½ years were used in the present experiments. They were ovariectomized when about 2 months old. The animals were kept in individual cages and fed evaporated milk and two different prepared dog's food at different times during the periods of observation. The dogs were weighed every two weeks. They were in excellent general health throughout the experiment.

Several series of observations were made on each dog. The estrogen used was estrone dissolved in oil of sesame (1 mgm. per cc.). Amounts of the oily solution were added to absolute alcohol so that a final concentration of 10 or 30 μg. of estrone were in 1 cc. of the alcoholic-oily solution. A solution of sesame oil in alcohol was applied as a control preparation. The total daily doses used ranged from 2 to 6 μg. of estrone.

The hair along the lateral body wall was clipped or in some experiments shaved. During some of the experiments the estrogen solution was applied to an area on one side of the body while the opposite side received the alcoholic solution of oil. One side thus served as a control for the other. After observations on the treated areas were completed the areas were again clipped or shaved so that the hair was of equal length on both. The experiments were then repeated reversing the order of treatment and control.

In other experiments larger areas were clipped or shaved so that the estrogen-treated and oil-treated areas were adjacent. The estrogen and control treatments then were applied to one-half of the denuded areas. After the effect of these treatments had been observed the hair was again clipped or shaved and the experiment continued by applying estrogen to areas previously treated with oil and vice versa.

The solutions were kept in tightly stoppered bottles to prevent evaporation and concentration. It was essential that the solution should not spread beyond the areas under study. Applications were made by dropping the solutions from 1 cc. tuberculin syringes fitted with needles. The solutions were applied slowly to prevent their spread beyond the areas desired. No attempt was made to prevent the dogs from licking the treated areas. The treatment produced no apparent irritation.

In order to determine whether or not the amounts of estrogen used might elicit a systemic reaction small amounts of estrogen (1.5 μg. in 0.05 cc.) were applied to each of the two nipples and immediately adjacent skin. Nipples on the opposite side were treated with the alcohol.

OBSERVATIONS

The local percutaneous application of dilute alcoholic solutions of estrone to recently shaved or clipped areas of the skin definitely re-

TABLE 1. SUMMARY OF TREATMENT AND OBSERVATIONS ON DOG #4

Treatment		Observations
Right side	Left side	
6/19/41 3 μg. E*		L. Complete recovery
8/19/41	.1 cc. A	R. Thin and short hair
9/15/41 (S)		L. Complete recovery R. Bald—further loss of hair since 8/19/41
11/11/41		Both sides completely recovered

*—Hair clipped.
A—Alcohol and oil applied
E—Estrone applied.
L. and R.—Refer to left and right sides.
(S) Treatment discontinued.

tarded the regrowth of hair at the sites of application (Tables 1 and 2). The inhibition of hair growth was limited to areas to which estrone was applied. This local effect was demonstrated by applying estrone to only a portion of the shaved area on a given side (Tables 3 and 4). The estrone-treated areas had not only fewer hairs, but those present were also shorter. Although definite differences were observed in the amount of hair present in the treated and untreated portions of the

TABLE 2. SUMMARY OF TREATMENT AND OBSERVATIONS ON DOG #6
(First experiments)

Date	Treatment		Observations
	Right side*	Left side	
12/10/40	C 3 μg. E	C 1 cc. A	
12/26/40			Both sides similar—slight growth
1/15/41			R. hair thin L. hair thick and longer than right
2/21/41			R. hair very thin, only few prime hairs L. recovery complete
4/ 7/41	C .2 cc. A	C 6 μg. E	R. hair still thin and short L. clipped area not apparent
6/19/41	C 6 μg. E	C .2 cc. A	R. thick but short hair L. very few long hairs—many stubs of clipped hairs
8/19/41			R. hair very thin and short L. thick but shorter than normal
9/ 5/41			R. hair thin—one bald area L. recovered

* C—Hair clipped.
A—Alcohol and oil applied.
E—Estrone applied.
L. and R.—Refer to left and right sides.

previously shaved area on the same side, a greater difference was evident when the estrone-treated areas were compared with the alcohol-treated sites on the opposite side. This greater side to side contrast was possibly due to spreading of the estrogenic solution over a considerable portion of a shaved control area on the homolateral side. The alcohol had no apparent effect on hair growth.

The most striking demonstration of the inhibitory effect of estrogen on hair growth was observed in dogs 3 and 6 (Tables 3 and 4) which were (1) shaved, (2) treated with estrogen for 44 days, (3) allowed to remain without further shaving or treatment for approximately 2 weeks, and (4) shaved again and observed without further treatment. On these dogs the areas to which estrone had been applied formerly remained bald for over 2 weeks after the final shaving, and hair growth was retarded at these sites for more than 4 weeks thereafter. Dog number 6 was shaved three times and subjected to two periods of estrone application between 12–12–'42 and 3–13–'43 (Table 4). Inhibition of hair growth at the sites of estrone application was observed subsequent to both the second and third shavings. In both dogs (#3 and #6) the two or more shavings followed by the daily application of alcohol in oil had no inhibitory effect on the regrowth of hair. If the estrone-treated areas were considered as a whole, regrowth of hair began first in those portions of the area most distal to the central site of application of the hormone. However, even in

TABLE 3. SUMMARY OF EXPERIMENTS IN DOG #3

Date	Position*	Treatment**		Observations	
		Right side	Left side	Right side	Left side
1/15/43	Ce	S 3 μg. E	S 0.3 cc. A	—	—
	Ca	S —	S —	—	—
2/ 4/43	Ce	Continued	Continued	Very slight growth	
	Ca			Growth—hair shorter than left	One half regrown
2/19/43	Ce	Continued	Continued	Slight regrowth	Good regrowth
	Ca			Good regrowth	Good regrowth
2/28/43	Ce	Stopped	Stopped	½ as much hair and shorter	Complete regrowth
	Ca			Shorter than on left	Complete regrowth
3/13/43	Ce	S	S		
	Ca	S	S		
3/22/43	Ce	—	—	Bald	Dense short hair
	Ca	—	—	Slight regrowth	Dense short hair
3/29/43	Ce	—	—	Bald	Continued growth
	Ca	—	—	Slight peripheral regrowth	Continued growth
4/16/43	Ce	—	—	Slight peripheral growth	One half regrowth
	Ca	—	—	More than in cephalic portion	One half regrowth
5/ 3/43	Ce	—	—	One half regrowth in number and length	Three-quarters regrowth in length
	Ca	—	—	Thicker than above	Same as above
5/12/43	Ce	—	—	Shorter and thinner than on left	Complete regrowth
	Ca	—	—	Shorter and thinner than on left	Complete regrowth

* Ce—Cephalic part of shaved area.
Ca—Caudal part of shaved area.
** S—Indicates the dates the areas were shaved.
E & A—See Table I.

these sites the hair grew more slowly and was less abundant than at untreated or alcohol-treated areas on the opposite side of the dog.

The percutaneous application for 44 days of an estrone solution resulted in a local inhibition of hair growth for over 2 months after the cessation of treatment. A second shaving demonstrated that the inhibitory effect of the estrogen on hair growth was still present even

TABLE 4. SUMMARY OF ESTROGEN EXPERIMENTS ON DOG #6 (SECOND EXPERIMENT)

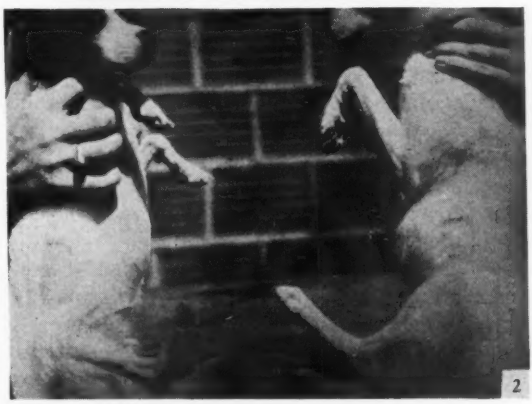
Date	Position*	Treatment**		Observations	
		Right side	Left side	Right side	Left side
11/12/42	Ce	C	C	—	—
	Ca	C+.2 cc. A	C+2 μg. E	—	—
11/28/42	Ce			90% regrowth	Same as right
	Ca			90% regrowth	Same as right
12/16/42	Ce			Complete regrowth	Same as right
	Ca	Treatment stopped	Treatment stopped	Complete regrowth	Same as right
1/15/43	Ce	S 3 μg. E	S .3 cc. A		
	Ca	S	S		
2/ 1/43	Ce	Continued	Continued	Good growth	Good growth
	Ca			Good growth	Good growth
2/19/43	Ce	Continued	Continued	Good growth	Good growth
	Ca			Almost complete regrowth	Thin, short hair
2/24/43	Ce	Continued	Continued	Hair shorter	Almost complete regrowth
	Ca			Almost complete regrowth	Almost complete regrowth
2/28/43	Ce	Stopped	Stopped	Short hair	Complete regrowth
	Ca			Complete regrowth	Complete regrowth
3/13/43	Ce	S	S		
	Ca	S	S		
3/22/43	Ce	—	—	Bare	Rapid growth
	Ca	—	—	Short thin hair	Rapid growth
4/16/43	Ce	—		Short thin hair	Rapid growth
	Ca	—		Intermediate regrowth	Rapid growth
5/ 3/43	Ce	—	—	About ½ regrowth in length	About 90% regrowth
	Ca	—	—	Continued growth	About 90% regrowth
5/12/43	Ce	—	—	All areas about equal	
	Ca	—	—	All areas about equal	

* Ce—Cephalic part of shaved area.
Ca—Caudal part of shaved area.
** S—Indicates the dates the areas were shaved.
A & E—See Table I.

after a complete regrowth of hair had taken place at the untreated areas.

There were no observable systemic reactions to the estrogen which was applied to the shaved areas. The genitalia and nipples showed no evidence of growth or vascular response to the estrogen applied to a small area on the side of the animal.

The local application of 1.5 μg. of estrone to each of two nipples of one dog when 5 months old produced a rapid growth of the nipples. The nipples on the opposite side treated with alcohol showed no growth and the external genitalia did not enlarge. The amount of



FIGS. 1 and 2. Dog #3 (on left in both photographs) and dog #6 (on right) had areas shaved on both sides on 1-15-'43 and were treated as follows until 2-28-'43: (1) On both dogs 0.3 cc. of estrone solution was applied daily (percutaneously) to the cephalic half of the shaved area on the right side; and (2) the same amount of alcohol was applied to the cephalic half of the shaved area on the left side. Both animals were shaved again on 3-13-'43 and there was no further treatment of either dog. The photographs show the appearance of these areas on the 40th day after cessation of treatment which was also the 27th day subsequent to the second shaving. Note that the estrone-treated areas (right side of dog #6 in upper photograph and right side of dog #3 in the lower) were bald whereas a very considerable amount of regrowth of hair had taken place on the alcohol-treated (left) sides. Careful examination shows that greater regrowth of hair was present in the caudal portions of the estrone-treated areas.

estrone absorbed was therefore too small to elicit a detectable systemic reaction even when applied to the thin abdominal skin. One dog was later treated in a similar manner when it was 10 months old. It was again observed that the estrogen-treated nipples increased rapidly in size while the alcohol-treated nipples showed no growth.

DISCUSSION

The direct response of the nipples and of the sexual cutaneous areas to estrogens were indicated by local response of one nipple of dogs or monkeys or of one area of sexual skin of monkeys that had received direct application of estrogens to these areas. Adjacent nipples or areas of sexual skin did not respond when the doses used were sufficiently small. The possibility that the hormone acted through the intermediary of some other endocrine gland was not indicated.

The injection of large amounts of estrogens into dogs which subsequently showed an inhibition of hair growth did not reveal information concerning the mechanism whereby these responses were attained. The present experiments in which the local application of estrogen inhibited the growth of hair on the treated areas yet failed to elicit any apparent systemic response indicated that the hormone acted directly. Had the pituitary or adrenal glands been involved all areas of the skin should have been similarly affected. This would also have been true had some product of estrogen inactivation or metabolism been the active substance. The doses active locally failed to elicit any evidence of changes in other reactive tissues, indicating that systemic levels of the hormone must have been very low.

The growth of the hair of puppies 4 months old and of dogs $2\frac{1}{2}$ years old were similarly inhibited. The season of the year did not alter the response except insofar as the rate of regrowth of hair on the control side was affected.

The prolonged inhibitory action of the estrogen, even after the cessation of injection, was revealed. The small amounts of estrogen did not completely inhibit the growth of hair, especially during the periods shortly after the start of the experiment. The difference between the control and estrogen-treated sides became more apparent as the experiments continued. This delayed and persistent effect was shown by the exaggerated inhibition of hair growth observed when the areas were again denuded. Whether the estrogen affected the hair follicles at certain stages of their development, or whether the result was due to a slow accumulation of estrogen in the skin to effective levels, was not determined.

Alopecia in man has been attributed to the action of androgens in individuals genetically susceptible to the unusual loss of hair (Hamilton 1942). In dogs alopecia or the partial inhibition of hair growth has been described in conditions in which an increased production of estrogens was indicated or when estrogens were administered (Greulich and Burford 1936; DeVita 1939). The condition was reversible after the inciting agent was discontinued, although as shown in the present experiments the recovery was somewhat delayed.

SUMMARY AND CONCLUSIONS

Small amounts (2 to 6 μg.) of estrone dissolved in oil and alcohol were applied locally to shaved or clipped areas of the skin on the lateral body walls of three different dogs. Each dog was used several times. Control areas on the opposite side received the oil-alcohol mixture.

Estrone so applied inhibited the rate of regrowth of hair in the treated areas. The partially restored hair of the estrone-treated areas, when again removed immediately subsequent to the cessation of estrogen-treatment, returned at a much slower rate than did the hair of the control areas.

Estrone in dogs apparently inhibited hair growth by a direct action on the skin or the hair follicles.

The amounts of hormone used were inadequate to stimulate growth of the nipples, and, hence, presumably inadequate to give a systemic response.

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THE CHEMICAL CHANGES IN RAT ADRENALS AFTER INJECTION OF β CHLOROETHYL VESICANTS[1]

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INJURY to the organism is invariably accompanied by hypertrophy of the adrenals (Tepperman, Engel, and Long, 1943). Since conclusions concerning the chemical changes taking place in hypertrophied adrenals have been based chiefly on histological evidence, the data for the protein, lipid, and water concentrations of normal and hypertrophied adrenals are limited. Decreases in the cholesterol concentration have been demonstrated after burns by Harkins and Long (1945) and after injection of the adrenotrophic hormone by Sayers, Sayers, Fry, White, and Long (1944) and by Sayers, Sayers, Tsang-Ying Liang, and Long (1946). Popjack (1944) analyzed the hypertrophied adrenals of shocked rats for water, lipids, and protein. He observed that the enlargement of the adrenals was due to an increased water content. The cholesterol ester concentration of the hypertrophied adrenals was markedly decreased.

During the course of experimental work with sulfur and nitrogen mustards, it was noted that the adrenals of treated rats were hypertrophied. In view of the limited data for chemical changes occurring in the hypertrophied adrenals after injury, studies were undertaken to determine the water, protein, total lipid, free and ester cholesterol and phospholipid concentrations of the adrenals of control and treated male rats.

METHODS

Inbred male rats of Wistar stock, about 70 days old and weighing between 150 and 200 gms., were used as experimental animals. The agents studied were injected into the exposed jugular vein of the anesthetized rat. The agents and their doses (LD 100) were: (a) bis (β chloroethyl) sulfide (0.4 mg./kg. in thiodiglycol); (b) ethyl bis (β chloroethyl) amine HCl (0.4 mg./kg.); (c) methyl bis (β chloroethyl) amine HCl (1.2 mg./kg.); and (d) tris (β chloroethyl) amine HCl (0.6 mg./kg.). The nitrogen mustards were dissolved in saline immediately before injection and about 0.25 cc. was injected into each animal. Control animals were injected intravenously with saline. All rats were starved after injections.

After exsanguination, the adrenals were removed and the adherent fat

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and connective tissue was dissected free. The glands were immediately weighed and treated for analysis. The determination of a given constituent was done on the pair of adrenals of each animal.

The water content was determined by drying the adrenals in vacuo over P_2O_5 at room temperature to constant weight. The total nitrogen of a pair of adrenals was estimated by a micro-Kjeldahl method. The cholesterol was extracted from the wet or dry adrenals with 5 cc. of an absolute alcohol:acetone mixture (1:1) under reflux for 3 hours. This solution was filtered and made up to a 10 ml. volume with alcohol:acetone mixture. Free and total cholesterol were determined on 5 and 3 ml. aliquots, respectively, by the Schoenheimer-Sperry method as described by Sperry (1938). Duplicate determinations were not possible. The total lipid C and phospholipid P was determined in duplicate on extracts prepared as follows: the adrenals were homogenized and extracted twice for 12 and 3 hours, respectively, with an absolute alcohol:acetone mixture (1:1). The extract was centrifuged free of solid particles, decanted and evaporated first on a hot plate and finally to dryness in vacuo. The residue was immediately extracted with 3 small portions of petroleum ether and made up to a 10 ml. volume An aliquot of this extract was analyzed for lipid C according to the procedure described by Van Slyke and Folch (1940). The factor 1.266 was used to convert C to total lipid. The petroleum ether extract was evaporated to dryness and the phospholipid P was determined by the method of Allen (1940). The factor 23.7 was used to convert P to phospholipid.

STATISTICAL TREATMENT

The standard error of the mean was determined by the formula $\sigma = \sqrt{\frac{d^2}{n(n-1)}}$ in which d is the deviation from the mean and n is the number of animals. The standard error of the difference between 2 means was calculated from the formula $[\sigma(\text{diff.})] = \sqrt{\sigma^2(\text{av.}_1) + \sigma^2(\text{av.}_2)}$ in which the $\sigma(\text{av.}_1)$ is the standard error of the first obtained average and $\sigma(\text{av.}_2)$ is the standard error of a second obtained average. Differences were considered significant when the difference between the means exceed three times $[\sigma(\text{diff.})]$.

RESULTS

Groups of rats injected with one of the 4 mustards were sacrificed at daily intervals. Very few of the animals survived more than 5 days. Data are recorded for adrenal weights, H_2O, total nitrogen, total lipids, total cholesterol, and phospholipids in terms of percentage concentration and mg./100 gm. body weight.

Adrenal Weight. The absolute weights of the adrenals of the saline control rats do not change appreciably during the 5 day period of starvation. The mean values for the absolute adrenal weights of the saline controls (Table 1) are as follows: 20, 24.8, 23.3, 22.7, 22.8, and 22.4 mg. on 0, 1, 2, 3, 4, and 5 days after saline injection, respectively. There appears to be a slight increase in adrenal weight on and after the first day of starvation. The data in Table 1 are expressed as mg.

TABLE 1. ADRENAL WEIGHTS

Days after inj.	Saline	Ethyl Bis (β Chloroethyl) Amine	Methyl Bis (β Chloroethyl) Amine	Tris (β Chloroethyl) Amine	Bis (β Chloroethyl) Sulfide
	mg./100 gm. body weight and standard error				
0	11.3 ± .24 (48)[x]				
1	14.8 ± .27 (38)	15.2 ± .22 (40)	15.7 ± .23 (46)	15.2 ± .31 (31)	15.6 ± .25 (39)
2	15.1 ± .22 (40)	17.1 ± .30* (49)	18.3 ± .20* (47)	16.6 ± .36* (43)	16.8 ± .29* (37)
3	16.1 ± .34 (37)	18.7 ± .47* (47)	21.8 ± .47* (41)	20.7 ± .37* (35)	19.5 ± .35* (38)
4	17.1 ± .33 (43)	17.7 ± .57 (35)	24.6 ± .54* (46)	21.7 ± .66* (34)	22.0 ± .77* (40)
5	17.1 ± .31 (38)	17.6 ± .51 (28)		22.8 ± .73* (28)	21.1 ± .76* (39)

[x] Figures in parenthesis represent number of animals.
* Represents significant difference from control saline value.

/100 gm. body weight and therefore reflect the loss in body weight. The mean adrenal weight/100 gm. rat and standard error for 48 untreated intact male rats is 11.3 ± 0.24 mg.; after 24 hours starvation, the mean value for 38 intravenously injected saline rats is 14.8 ± 0.27 mg. The remaining values for the experimental period are also significantly greater than those of the control.

After injection of the mustards, the absolute weights of the adrenals increase. This increased weight is reflected in the mean adrenal weights/100 gm. body weight. The loss of body weight in the starving mustard-treated rats is about the same as that encountered in the saline controls. Most of the values for adrenal weight in the mustard animals are significantly greater than for the respective saline controls excepting on the first day after injection. It was also observed that the adrenals hypertrophy after injecting sublethal doses of these agents.

Water (Table 2). The percentage concentrations of water of the adrenals of saline injected rats remain constant during starvation. Slight but significant increases are seen in many of the mustard treated rats. Since the adrenals of the experimental animals are hypertrophied, the absolute amounts of water are greater. Half of the mean values per 100 gm. body weight of the experimental animals are significantly greater than their respective saline controls. All values on the third day are significantly increased.

TABLE 2. WATER

	mg./100 gm. body weight and standard error				
0	7.8 ± .24 (8)[x]				
1	9.6 ± .31 (10)	10.6 ± .29 (10)	10.8 ± .30 (10)	9.4 ± .38 (10)	11.2 ± .27* (12)
2	10.3 ± .35 (10)	12.2 ± .39* (20)	12.8 ± .38* (10)	11.4 ± .44 (20)	10.8 ± .29 (9)
3	10.6 ± .38 (10)	13.9 ± .95* (9)	14.4 ± .57* (10)	13.4 ± .28* (10)	14.5 ± .68* (8)
4	12.0 ± .55 (8)	14.8 ± .98* (6)	15.8 ± .63* (8)	14.1 ± .98 (8)	12.7 ± 1.00 (5)
5	11.0 ± .21 (10)	11.5 ± .60 (6)		15.2 ± .71* (9)	13.1 ± 1.53 (5)
	percentage concentration				
0	66 (62–68)†				
1	69 (68–70)	71 (69–72)	71 (69–73)	67 (65–70)	69 (65–71)
2	68 (65–70)	71 (69–75)	71 (69–72)	71 (64–73)	68 (66–70)
3	68 (65–70)	69 (64–74)	72 (69–75)	69 (66–71)	72 (71–73)
4	67 (65–70)	70 (67–76)	70 (66–73)	69 (67–71)	68 (66–70)
5	67 (65–69)	67 (66–68)		68 (67–69)	65 (62–67)

[x] Figures in parenthesis represent number of animals.
* Represents significant difference from control saline value.
† Mean (Minimum and Maximum) values.

TABLE 3. PROTEIN

Days after inj.	Saline	Ethyl Bis (β Chloroethyl) Amine	Methyl Bis (β Chloroethyl) Amine	Tris (β Chloroethyl) Amine	Bis (β Chloroethyl) Sulfide
	mg./100 gm. body weight and standard error				
0	2.48±.07 (9)[x]				
1	2.56±.07 (9)	2.85±.05* (10)	3.01±.12* (10)	2.80±.12 (10)	2.69±.08 (10)
2	2.81±.07 (10)	3.23±.06* (8)	3.41±.08* (10)	3.14±.07* (9)	3.52±.07* (8)
3	2.72±.12 (9)	3.28±.15* (18)	3.96±.20* (9)	3.66±.13* (7)	3.84±.10* (9)
4	3.20±.11 (9)	3.40±.17 (14)	3.76±.18 (11)	3.98±.17* (10)	4.18±.29* (13)
5	3.02±.11 (10)	3.09±.14 (9)		4.60±.57* (4)	3.35±.11 (12)
	percentage concentration				
0	19.5 (18.5–22.0)†				
1	18.7 (17.0–20.0)	18.4 (18.0–20.0)	17.7 (16.5–19.0)	18.1 (17.0–19.0)	16.6 (14.5–18.5)
2	18.9 (17.0–20.0)	18.2 (17.5–19.5)	17.7 (17.0–20.0)	18.6 (17.0–19.5)	19.0 (18.0–20.0)
3	18.5 (16.5–20.5)	18.0 (17.0–19.0)	17.3 (15.5–19.0)	17.6 (17.0–18.5)	18.0 (17.0–20.0)
4	18.8 (17.5–19.5)	17.6 (16.0–19.5)	17.3 (16.2–18.0)	17.6 (17.0–18.5)	17.5 (16.0–19.5)
5	18.1 (17.5–18.5)	17.2 (14.5–19.0)		15.8 (15.0–17.0)	17.5 (16.0–19.0)

[x] Figures in parenthesis represent number of animals.
* Represents significant difference from control saline value.
† Mean (Minimum and Maximum) values.

Protein (Table 3). The percentage concentration of the adrenal protein (N×6.3) remains fairly constant in the saline-injected rats during starvation. After injection of the respective agents, these values tend to diminish and probably reflect a dilution effect. In general, most of the mean values for the mg. adrenal protein/100 gm. body weight are significantly greater than the controls.

Total Lipids (Table 4). The percentage concentrations of total lipids decrease on the first day of starvation in the saline-injected rats and increase slightly above the control level on succeeding days. After injection of the agents, the total lipid concentrations are markedly decreased on the 2nd, 3rd, and 4th days for the nitrogen mustards and on the 3rd, 4th, and 5th days for the sulfur mustard. The lowest values were obtained in the methyl bis (β chloroethyl) amine group regardless of whether the administered dose was lethal or sublethal.

The data for total lipid per 100 gm. rat are variable. Many of the

TABLE 4. TOTAL LIPID

Days after inj.	Saline	Ethyl Bis (β Chloroethyl) Amine	Methyl Bis (β Chloroethyl) Amine	Tris (β Chloroethyl) Amine	Bis (β Chloroethyl) Sulfide
	mg./100 gm. body weight and standard error				
0	1.50±.13 (8)[x]				
1	1.77±.08 (9)	2.02±.07 (10)	1.90±.06 (18)	2.28±.13* (9)	1.69±.05 (7)
2	2.13±.08 (10)	1.78±.09* (10)	1.82±.09* (18)	2.07±.12 (10)	2.42±.13 (10)
3	2.63±.14 (8)	2.27±.07 (10)	2.24±.13 (9)	2.11±.06* (10)	1.94±.10* (9)
4	2.21±.07 (16)	2.16±.09 (9)	2.75±.08* (19)	2.65±.15 (9)	2.26±.09 (7)
5	2.89±.12 (9)	2.20±.08* (8)		3.41±.13* (9)	2.75±.12 (10)
	percentage concentration				
0	13.6 (11.2–15.5)†				
1	11.7 (10.4–13.5)	13.1 (11.2–14.8)	12.3 (9.9–14.9)	13.9 (11.1–16.7)	11.3 (10.8–11.8)
2	14.8 (13.9–16.3)	10.4 (8.3–12.8)	10.7 (8.6–13.8)	11.6 (7.7–15.9)	14.0 (12.4–15.8)
3	16.4 (14.7–18.6)	12.5 (10.8–14.1)	10.7 (8.9–11.5)	10.0 (8.7–12.2)	10.0 (8.7–11.1)
4	13.7 (10.3–16.5)	12.7 (10.0–14.6)	10.7 (8.0–15.1)	11.1 (8.2–13.4)	11.9 (10.0–14.4)
5	16.1 (13.8–20.0)	13.9 (13.2–16.2)		14.7 (13.4–16.5)	13.2 (10.6–17.4)

[x] Figures in parenthesis represent number of animals.
* Represents significant difference from control saline value.
† Mean (Minimum and Maximum) values.

TABLE 5. TOTAL ADRENAL CHOLESTEROL

Days after inj.	Saline	Ethyl Bis (β Chloroethyl) Amine	Methyl Bis (β Chloroethyl) Amine	Tris (β Chloroethyl) Amine	Bis (β Chloroethyl) Sulfide
		mg./100 gm. body weight and standard error			
0	.284 ± .02 (12)[x]				
1	.334 ± .03 (10)	.218 ± .01* (10)	.291 ± .02 (10)	.215 ± .02* (12)	.278 ± .02 (9)
2	.392 ± .03 (10)	.270 ± .03* (11)	.224 ± .03* (9)	.169 ± .02* (14)	.401 ± .03 (10)
3	.473 ± .03 (10)	.234 ± .09 (10)	.252 ± .06* (10)	.134 ± .01* (18)	.257 ± .03* (11)
4	.558 ± .04 (10)	.512 ± .03 (12)	.520 ± .07 (10)	.339 ± .03* (16)	.220 ± .04* (15)
5	.521 ± .03 (10)	.581 ± .03 (11)		.721 ± .04* (16)	.639 ± .09 (10)
		percentage concentration			
0	2.6 (1.9–3.1)†				
1	2.0 (1.1–2.8)	1.5 (1.2–1.7)	1.9 (1.4–2.4)	1.5 (1.0–2.4)	1.9 (1.2–2.7)
2	2.4 (1.7–3.0)	1.6 (0.6–3.0)	1.1 (0.6–1.9)	1.1 (0.4–2.9)	2.5 (2.0–3.2)
3	2.7 (2.1–3.5)	1.3 (0.6–2.3)	1.2 (0.6–2.9)	0.7 (0.4–1.5)	1.4 (0.8–2.5)
4	3.1 (2.4–4.0)	3.3 (1.1–4.8)	1.9 (0.8–3.5)	1.8 (0.5–3.6)	1.2 (0.4–3.6)
5	3.0 (2.2–3.5)	3.1 (2.9–3.6)		3.4 (2.5–4.4)	3.2 (0.4–5.3)

[x] Figures in parenthesis represent number of animals.
* Represents significant difference from control saline value.
† Mean (Minimum and Maximum) values.

values are lowered on the second and third days after injection of the nitrogen mustards and on the third day after sulfur mustard. On the 4th and 5th days, the lipid values return to the control range or are increased. These data differ from the results for percentage concentration and reflect the influence of the adrenal hypertrophy.

Total Cholesterol (Table 5). Cholesterol comprises approximately 20% of the adrenal total lipids in the saline controls. The percentage concentration of cholesterol remains constant in the starving control group; the values for mg./100 gm. body weight increase due to the loss of weight during starvation.

After injection of the 4 mustards, both the percentage concentration and the mg./100 gm. body weight of adrenal cholesterol undergo marked changes. The tris (β chloroethyl) amine group shows the greatest changes from the controls. The general picture appears to be one of a sharp decline during the first 3 days followed by a return to or an increase above the control levels.

TABLE 6. PHOSPHOLIPID

Days after inj.	Saline	Ethyl Bis (β Chloroethyl) Amine	Methyl Bis (β Chloroethyl) Amine	Tris (β Chloroethyl) Amine	Bis (β Chloroethyl) Sulfide
		mg./100 gm. body weight and standard error			
0	.40 ± .01 (15)[x]				
1	.64 ± .02 (9)	.66 ± .02 (10)	.61 ± .02 (15)	.59 ± .02 (10)	.61 ± .02 (7)
2	.59 ± .02 (7)	.74 ± .04* (10)	.73 ± .02* (17)	.73 ± .02* (10)	.73 ± .02* (10)
3	.64 ± .05 (8)	.80 ± .05 (10)	.98 ± .06* (9)	.94 ± .04* (10)	.86 ± .05* (9)
4	.60 ± .04 (16)	.73 ± .05 (7)	1.10 ± .04* (18)	.86 ± .08* (9)	.81 ± .06* (6)
5	.76 ± .03 (9)	.60 ± .02* (8)		1.00 ± .07* (11)	
		percentage concentration			
0	3.8 (3.5–4.1)†				
1	4.3 (4.0–4.6)	4.2 (4.0–4.4)	4.0 (3.3–5.3)	4.0 (3.6–4.3)	4.0 (3.8–5.0)
2	4.0 (3.8–4.4)	4.3 (4.2–4.4)	4.2 (3.7–5.6)	4.1 (3.7–4.5)	4.2 (3.9–4.5)
3	3.9 (3.5–4.3)	4.4 (3.9–5.0)	4.5 (3.9–4.8)	4.4 (4.0–4.9)	4.4 (3.9–5.0)
4	3.6 (2.6–4.3)	4.2 (4.0–4.6)	4.2 (3.5–4.7)	4.1 (3.4–4.9)	4.1 (3.8–4.4)
5	4.2 (3.9–4.4)	3.8 (3.2–4.1)		4.0 (3.6–4.6)	

[x] Figures in parenthesis represent number of animals.
* Represents significant difference from control saline value.
† Mean (Minimum and Maximum) values.

Data for free and bound cholesterol are graphically presented in Figure 1. It is striking that the free cholesterol content of the adrenals remains within the control range in practically all groups. Severe injury does not affect the free cholesterol concentration of the adrenals. However, the ester cholesterol of the adrenals shows marked variations. It is clear that decreases in total cholesterol content are due to the changes in the bound cholesterol fraction. The nature of these changes certainly demonstrates the lability of these esters. The mean

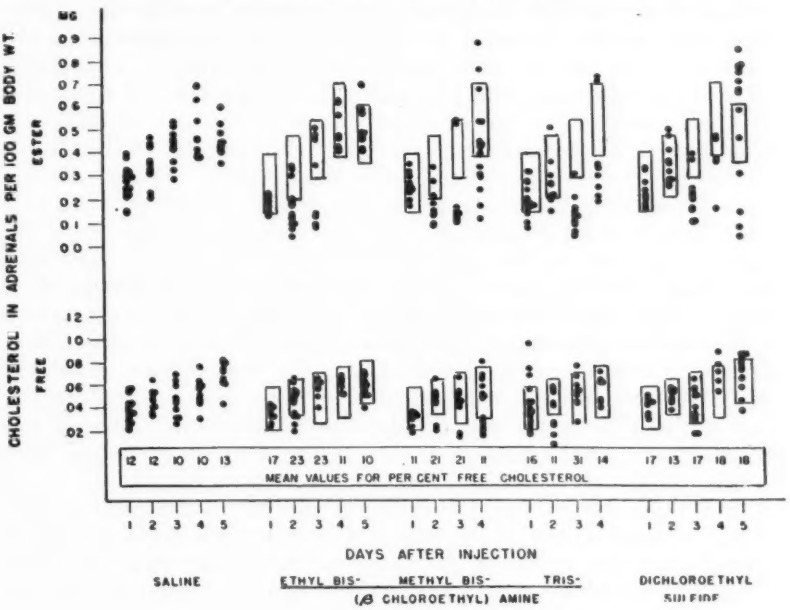

FIG. 1

values for the per cent of free cholesterol which are shown in Figure 1 also reflect the large decreases in the concentration of bound cholesterol.

Phospholipids (Table 6). These constituents comprise approximately 25% of the total lipids. The mean percentage concentration of the adrenal phospholipids is not affected after intoxication. Consequently, the mg./100 gm. body weight of these phospholipids are increased in the hypertrophied adrenals, and small but significant differences are obtained in most of the treated animals after the first day.

The sum of the percentage concentrations of water, protein, and total lipid for each group should yield approximately 100%. When the results were treated in this manner, values between 97 and 103 per cent were obtained. The sum of the milligrams of water, protein,

and total lipids per 100 gm. body weight approximates the weights of the respective adrenals.

DISCUSSION

Selye (1937) first stressed the effect of noxious stimuli on the hypertrophy of the adrenal cortex. The hypertrophy of the adrenals which occurs after injecting the β chloroethyl vesicants does not appear to be a specific response to these agents but rather a reaction typical of the general adaptation syndrome for non-specific stress. From the results presented in this paper, the enlargement of the adrenals is due chiefly to an increase in water content and to some extent to proteins and phospholipids. The congestion and dilatation of small vessels observed in these adrenals are not of sufficient magnitude to account for the degree of hypertrophy noted (Kindred).

Sayers, Sayers, Fry, White, and Long (1944), Sayers, Sayers, Lewis, and Long (1944), and Sayers, Sayers, Tsang-Ying Liang, and Long (1946) showed that the injection of adrenotrophic hormone causes a decrease in the adrenal cholesterol and ascorbic acid concentrations. On the basis of their results and those of others, they suggest that cholesterol and ascorbic acid may serve as precursors of steroid cortical hormones. Popjack (1944) presented evidence that the bound cholesterol is decreased and the steroid hormone is increased in the adrenals of shocked rats. This worker supports the view of the Yale group that the decreased cholesterol content is associated with the formation of corticosterone or other steroid hormones.

The evidence for the conversion of adrenal cholesterol to hormones is based on indirect evidence. If this hypothesis is correct, it follows from the evidence presented in this paper that esterified cholesterol would serve as the precursor of such hormones. During the first 4 days after injection of the vesicants, the bound cholesterol content of the adrenals is decreased to varying degrees in all but one group. It should be pointed out that the adrenals contain a higher percentage of bound cholesterol than any other tissue or body fluid.

According to Popjack (1944), "The very marked decrease in ester cholesterol content after crushing injury indicates that the lipoid depletion of the adrenal cortex observed histologically is a cholesterol depletion; conversely, it can be concluded also that a very large proportion of the stainable lipid in the adrenal cortex is bound cholesterol." An analysis of the absolute changes of total lipid and total cholesterol is presented in Figure 2 in order to determine the relationship of these two constituents. It is probable that the animals are at the end of the counter-shock phase of the "alarm reaction" (Selye, 1940) on the third day after injection of the mustards. It can be seen that the changes in the lipid content of the adrenals are not dependent on the cholesterol content during the first 2 days. The most

marked and consistent decreases in total lipids are obtained on the third day for all groups. At this time, the total cholesterol content is responsible for 66, 57, 65, and 32 per cent, respectively, of the decrease in total lipid content. The decrease in total lipid at this time is due largely to the loss of the ester cholesterol.

Animals injected with LD 100 doses of the mustards become very ill on the fourth and fifth days and usually die at this time. This

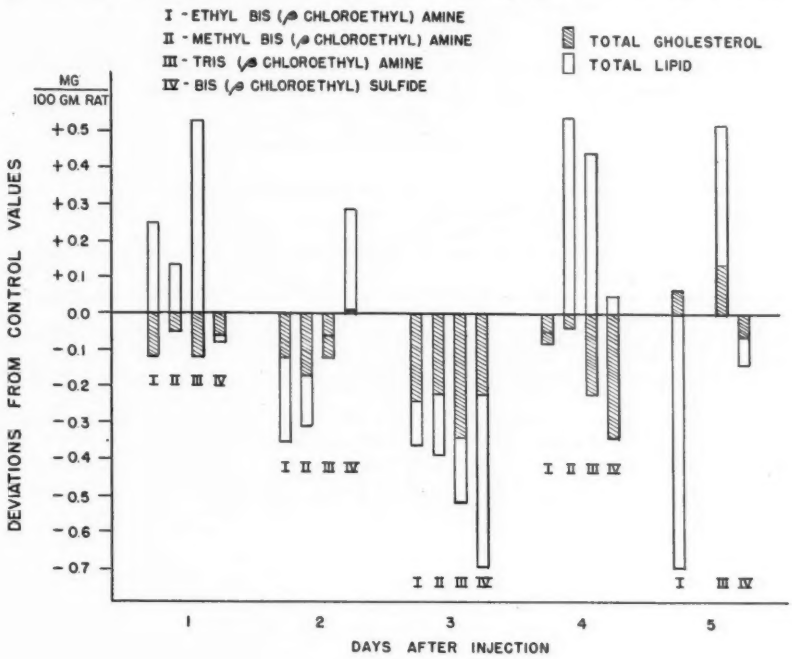

FIG. 2

would correspond to the "stage of exhaustion" according to Selye (1944). At this time, the total lipid contents show marked variations which do not appear to be related to the total or bound cholesterol. It is also interesting to point out that the greatest variations in the content of ester cholesterol are noted on the 4th and 5th days (Figure 1).

Since a consistent relationship between total lipid and total cholesterol is observed only on the third day after injection, it is believed that these two constituents are independent of each other in their response to stress.

SUMMARY

The adrenals of rats injected intravenously with LD 100 doses of 4 β chloroethyl vesicants were analyzed for water, protein, total lipid, free and bound cholesterol and phospholipid. The administration of

these noxious agents caused a marked hypertrophy of the adrenals. Data are presented which show that adrenal enlargement is due chiefly to an increased water content and to some extent to protein and phospholipid. The total cholesterol concentration of the adrenals is markedly decreased due to the loss of ester cholesterol. The total lipid concentration is decreased. The changes are not dependent on the variations in the cholesterol or phospholipid concentrations.

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THE CHEMICAL CYTOLOGY OF THE RAT'S ADRENAL CORTEX IN PANTOTHENIC ACID DEFICIENCY

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IN PRELIMINARY OBSERVATIONS on puppies with severe pantothenic acid deficiency, we noted that their adrenal cortices exhibited a depletion of lipids. Investigators in many laboratories have reported hemorrhage and necrosis in the adrenal cortices of rats on similarly deficient diets (Daft and Sebrell, 1939; Nelson, 1939; Ashburn, 1940; Miller, Shaw, Elvehjem and Phillips, 1940; Morgan and Simms, 1940; Salmon and Engel, 1940; and Supplee, Bender and Kahlenberg, 1942). Furthermore, in pantothenic acid deficient puppies Schaefer, McKibbin and Elvehjem (1942) found alterations in blood chloride, glucose, and non-protein nitrogen, in addition to gastritis and hemorrhagic kidneys and thymuses. All of these changes have been recorded by Selye (1937) and others as characteristic of an "alarm reaction" resulting from any of a variety of stress conditions.

Because of these observations we decided to make a detailed study of the adrenal cortex of rats during the development of pantothenic acid deficiency in the hope of determining whether the cortex is involved early in the deficiency or only secondarily. Moreover, histochemical methods for the demonstration of ketosteroids (Dempsey and Wislocki, 1946) made it possible to estimate the amount of the cortical hormones in the gland. In addition, the thymus, kidney and liver, described as pathological in the earlier studies, were reexamined by appropriate methods. And, finally, as an additional means of gauging metabolic changes in the deficient animals, their blood was analyzed for glucose and non-protein nitrogen. By these methods we hoped to gain information regarding the site or sites of action of pantothenic acid in the mammalian body. Because the rat is susceptible to pantothenic acid deficiency and since a detailed study of its adrenal cortex was already in progress (Deane and Greep, 1946), it was chosen for the present investigations. In order to pro-

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duce a deficiency of this ubiquitous vitamin, an entirely purified diet was essential. The control diet, although adequate for growth, was found to result in certain abnormalities to be described later.

MATERIALS AND METHODS

Thirty-two male albino rats of about thirty days of age, purchased from Rockland Farms, were separated into two groups. Thirteen of these animals in the control group were fed a purified ration ad libitum. The ration consisted of sucrose—72%, purified casein (SMA)—18%, salts (Phillips and Hart)—4%, corn oil—3%, cod liver oil—3%; plus riboflavin—400 mcg. per 100 gms. ration, thiamin chloride—100 mcg., pyridoxine hydrochloride—200 mcg., nicotinic acid—1 mg., choline chloride—200 mg., and calcium pantothenate—1 mg. The remaining nineteen animals were subjected to marked pantothenic acid deficiency by being placed on the above purified ration minus calcium pantothenate.

Generally two animals from the control group and three from the deficient group were killed weekly throughout the experimental period of six weeks (the animals are listed on the first 2 figures). In the case of the deficient animals a deliberate attempt was made each time to select one that that was minimally affected and one that was severely affected, as judged by growth and other symptoms. The animals were always killed in the afternoon. At the time that each animal was killed, it was bled from the heart under deep nembutal anesthesia. The blood which was collected was analyzed chemically for non-protein nitrogen and glucose by the methods of Folin and Wu (1919) and Hoffman (1937) respectively. The wet weights of the paired adrenals and of the thymuses were determined on a Roller-Smith precision balance.

The following histochemical and cytological procedures were carried out on adrenals, liver and kidney. 1) These organs were fixed in 10% neutral formalin and sectioned on a freezing microtome at 15 μ. Sections were stained with sudan IV, sudan black B, the Schiff reagent, and 2-4 dinitrophenylhydrazine. Another section was mounted unstained for observation with the polarizing and fluorescence microscopes. Control sections for the last and for the Schiff preparation were first extracted with acetone. 2) These organs were also fixed in Maximow's Zenker-formalin, postchromated, sectioned at 3 μ, and stained for mitochondria with iron-alum hematoxylin or Mallory's phosphotungstic-acid hematoxylin. 3) A piece of liver was fixed in picro-alcohol-formalin, sectioned at 5 μ, and stained for glycogen by the Bauer-Feulgen method.

ANATOMICAL OBSERVATIONS

Good growth occurred on the control ration (Fig. 1), whereas growth was impaired in the rats maintained on the deficient ration (Fig. 2). The animals on the deficient diet ceased to gain weight and generally developed diarrhea. In addition porphyrin deposits appeared on their whiskers and their hair became rusty. At necropsy their intestines were distended with gas and their adrenals, thymuses and kidneys had a mottled appearance which was attributable to hemorrhage.

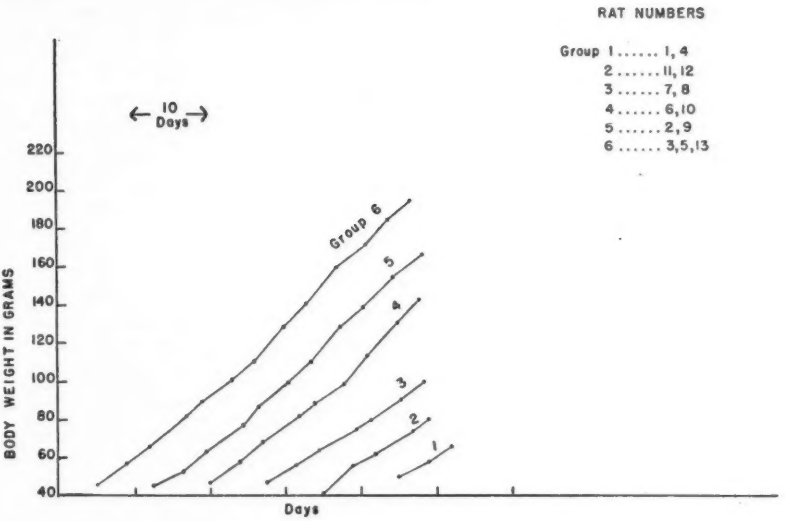

FIG. 1. Growth curves for rats on the control purified diet. The groups were killed at the end of 1, 2, 3, 4, 5, and 6 weeks respectively.

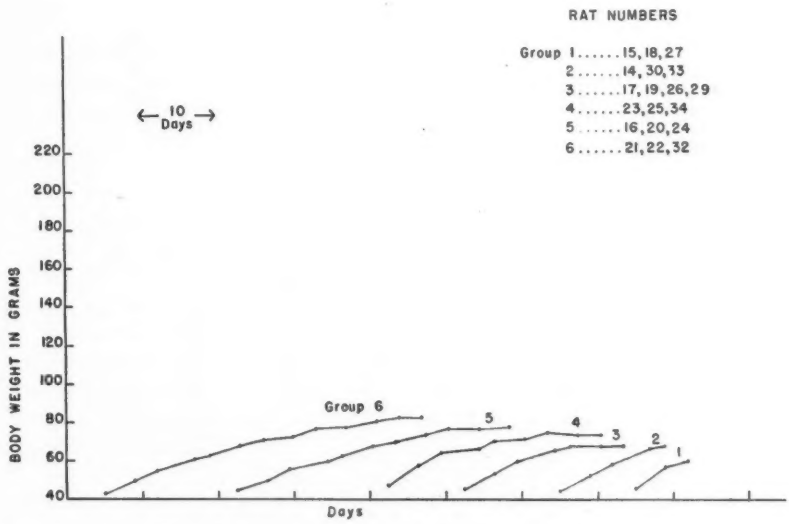

FIG. 2. Growth curves for rats on the purified diet deficient in calcium pantothenate. Groups killed concomitantly with those listed on figure 1.

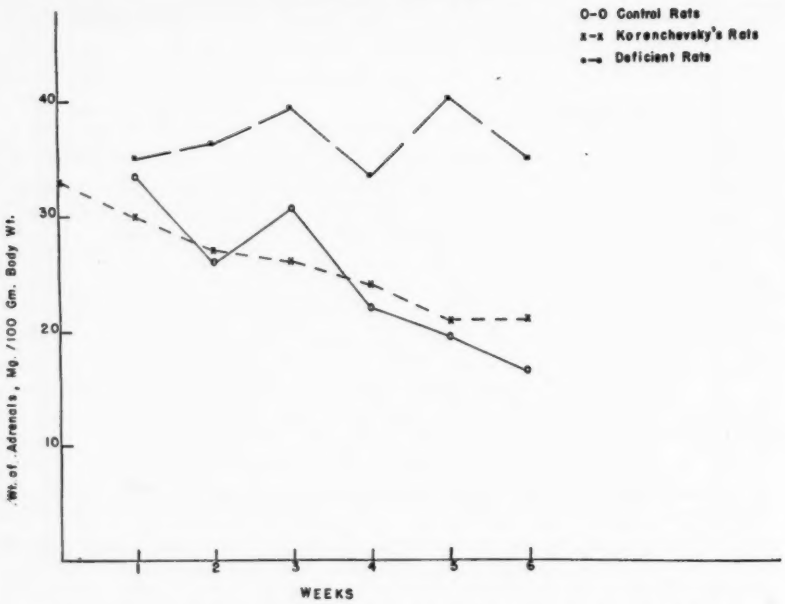

FIG. 3. Changes in the average relative weights of the paired adrenals of the control rats and of the deficient rats for the six week experimental period, and of normal rats from 30 days of age (Korenchevsky, 1942).

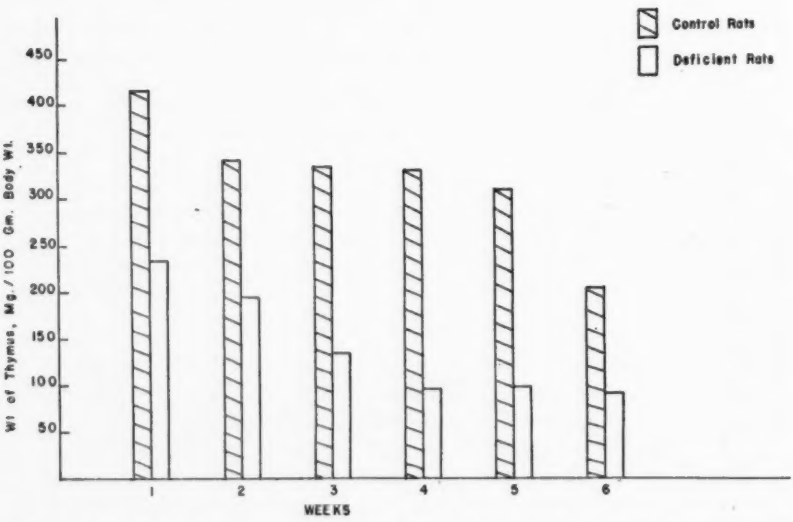

FIG. 4. Changes in the average relative weights of thymuses of the control and deficient rats for the six week experimental period.

The weights of the adrenals in proportion to body weight for the two groups of rats are presented in figure 3. Also included are the proportional weights of the adrenals of normal rats of the same age as reported by Korenchevsky (1942). The relative weights of the adrenals of the control animals decreased with age much as did those of Korenchevsky's rats, but those of the deficient animals remained high throughout the experimental period.

The relative weights of the thymuses are presented in figure 4. The data indicate that the weights of the thymuses of the control group on the control diet were within the normal range (Donaldson, 1924, p. 263), whereas those of the experimental animals underwent immediate atrophy.

In order to detect any change in the relative size of the cortex of the adrenal glands, the area of a mid cross-section of each adrenal, magnified 56 times, was measured with a planimeter and the area of the medulla subtracted, thus giving the area of the cortex; the actual

TABLE 1. THE AVERAGE PROPORTION OF THE AREA OF THE MID CROSS-SECTION OF THE CORTEX TO THE WEIGHT OF THE ADRENAL, IN SQ. MM./MG., FOR THE CONTROL AND DEFICIENT GROUPS OF RATS

Group	Control rats	Deficient rats
1	.155	.125
2	.155	.117
3	.115	.114
4	.121	.160
5	.132	.148
6	.122	.147

area of cortical material was then calculated. Since the adrenals were of differing sizes, it was necessary to divide the area by the weight of the gland in order to obtain the proportion of the cortex to the whole gland. When this was done, it became apparent that the cortex comprised a smaller proportion of the gland in the deficient rats than in the controls during the first two weeks of the experiment. After this the relationship became reversed so that the cortex represented a greater proportion of the glands in the deficient rats than in the controls (Table 1). These findings in the adrenal glands are evident by comparison of figures 7, 9, 10, and 11. Thus, the width of the cortex of a rat maintained on the deficient diet for 1 week (Fig. 9) is less than that of a control (Fig. 7), while that of an animal kept on the deficient diet for 4 weeks (Fig. 10) is equal to that of the control, and finally, that of an animal kept for 6 weeks (Fig. 11) is greater than that of the control.

CHEMICAL OBSERVATIONS

The average blood-glucose and non-protein nitrogen of the control and pantothenic acid deficient rats are shown in table 2. No significant differences can be seen between the bloods of the control and

deficient rats in respect to these two blood constituents, with the possible exception of the final upswing in NPN in the deficient rats.

HISTOLOGICAL OBSERVATIONS

The adrenal cortex in the control rats. Histological examination of the lipoid and mitochondrial preparations of the adrenals of the control rats revealed that without exception the cortex was normal in appearance. Since the animals were young, there were only two definite zones, a zona glomerulosa and a broad zona fasciculata extending without noticeable interruption to the border of the medulla (Fig. 7). In these adrenals there was, if anything, more lipid than in those of rats maintained on dog chow (cf. Deane and Greep, 1946, Fig. 5). As in the previous series, the lipoid droplets were most numerous in the cells of the glomerulosa and the outer fasciculata, with fewest

TABLE 2. THE AVERAGE AMOUNTS OF GLUCOSE AND NON-PROTEIN NITROGEN IN MILLIGRAMS/100 CC. IN THE BLOOD OF THE CONTROL AND DEFICIENT GROUPS OF RATS

Group	Control rats		Deficient rats	
	Glucose	NPN	Glucose	NPN
1	140	—	142	—
2	140	66.5	156	47.0
3	152	61.0	151	54.0
4	183	48.0	179	32.0
5	212	72.0	160	68.0
6	—	49.0	—	127.0

in the transitional region between the glomerulosa and fasciculata as well as in the inner fasciculata. This lipid exhibited the several reactions which indicate the presence of ketosteroids; thus, it gave the Schiff plasmal reaction (Fig. 9), formed a yellow phenylhydrazone after treatment with phenylhydrazine, showed a greenish-white autofluorescence, and revealed typical birefringence on the polarizing microscope (Fig. 5).

In hematoxylin preparations the mitochondria appeared as fine granules in the cells of the glomerulosa and as larger, more irregular granules in the spongy part of the fasciculata. In the latter region there were also many liposomes, which are lipid-containing droplets that survive paraffin imbedding after appropriate fixation and stain with hematoxylin (Hoerr, 1936). These bodies are presumably related to the secretory products of this zone.

The adrenal cortex in the deficient rats. Preparations of the adrenals of the deficient rats revealed the gradual loss of sudanophil lipids from the zona fasciculata. Paripassu with the disappearance of the lipoid material from the inner part of the fasciculata this region gradually assumed the appearance of a zona reticularis. By the end of the first week all lipoid droplets had disappeared from the juxtamedullary reticularis (Fig. 9); by the end of four weeks they were gone from the

inner fasciculata as well (Fig. 10), and by the end of six weeks from the whole fasciculata (Fig. 11). Concomitant with this recession of the lipoid droplets, there was a reduction of the lipid in the more peripheral zones as well, watery vacuoles making their appearance in the cells (Figs. 10 and 11). In addition, small fat droplets were frequently observed in the lumina of the sinusoidal capillaries of the

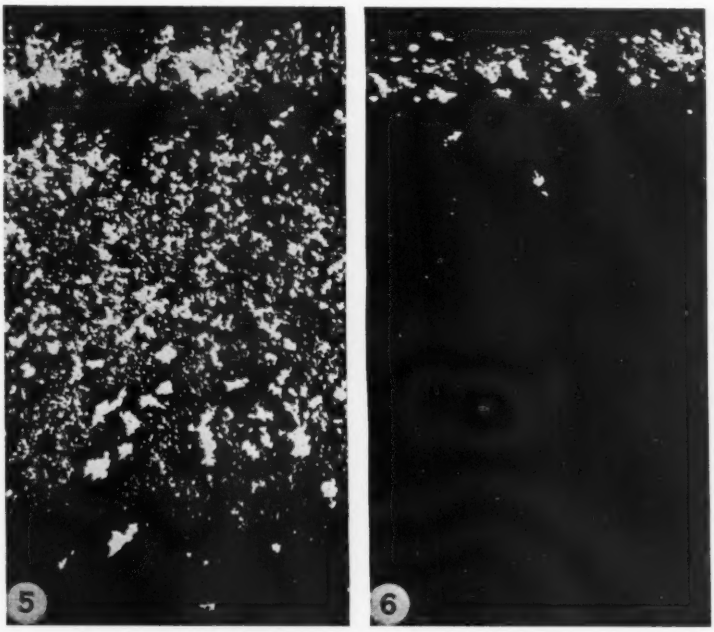

FIG. 5. Adrenal cortex of rat on control diet for 5 weeks (#9). Gland fixed in 10% neutral formalin, frozen section cut at 15μ. Photographed on polarizing microscope. Large amounts of birefringent lipoid both in zona glomerulosa and whole fasciculata, none in transitional zone between glomerulosa and fasciculata nor in inner fasciculata. Birefringent particles both coarse and fine. ×130.

FIG. 6. Adrenal cortex of rat on pantothenic-acid deficient diet for 5 weeks (#24). Similar preparation. Maintenance of birefringent lipoid in zona glomerulosa but complete disappearance from fasciculata. ×130.

cortex as though the droplets had just been secreted by the cortical cells.

The ketosteroid reactions disappeared even more abruptly than did the sudanophilia, as demonstrated by the fact that the tests for ketosteroids were entirely negative in the reticularis and inner fasciculata and only faintly positive in the outer fasciculata by the end of only one week. At the same time the finer birefringent particles had disappeared from the reticularis and fasciculata. The reduction

of birefrigent material was continuous so that by the end of six weeks all birefringent lipoid material had vanished from the fasciculata (Fig. 6). Thus, in rats maintained on a pantothenic acid deficient diet all of the adrenal cortex, with the exception of the zona glomerulosa, becomes rapidly and completely exhausted of ketosteroid.

The hematoxylin preparations of the adrenals of the deficient animals showed a steady deterioration of the cortex. These alterations were limited to the inner zones, with no visible changes occurring in the zona glomerulosa. The cells of the latter zone were in all instances filled with a normal complement of tiny fat vacuoles; and the usual small, granular mitochondria lay in the protoplasmic interstices.

In the fasciculata, however, there were marked histological changes. During the first two weeks of the deficiency many mitotic figures made their appearance in the outer fasciculata. And as early as one week a typical reticularis began to develop, formed seemingly as a result of a disorganization and rearrangement of the cells of the innermost part of the zona fasciculata. These changes led gradually to the formation of a broad reticular zone consisting of compact, darkly staining cells, many of which were dying. From the second week on, foci of complete necrosis and of hemorrhage appeared in both the reticularis and fasciculata, followed in the more advanced stages of the deficiency by fibrous infiltration of the zona reticularis. The most extreme example of a disintegration of the fasciculata was presented by the adrenals of rat #16, after 5 weeks of the deficiency. In a photomicrograph of one of its adrenals, the line of demarcation between the fairly viable cortical cells and the necrotic, moribund ones has been drawn in (Fig. 12), and one can readily see how much the functioning cortex had been reduced.

In addition to these relatively gross changes, cytological indica-

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EXPLANATION OF PLATE 1, FIGS. 7–11

Adrenal cortices of rats. Glands fixed in 10% neutral formalin, frozen sections cut at 15μ. All photographs 105×.

FIG. 7. Rat on control diet 4 weeks (#6). Section stained with sudan IV and hematoxylin, photographed with blue filter. Crowded lipoid droplets in zona glomerulosa, outer fasciculata and inner fasciculata, fewer in transitional zone between glomerulosa and fasciculata and in juxtamedullary zone (reticularis). Even more lipid than in rats on dog chow (cf. Deane and Greep, 1946, figs. 5, 9, 11, 16).

FIG. 8. Rat on control diet for 4 weeks (#6). Section stained by the Schiff plasmal reaction; photographed with combined green and yellow filters. Lipoid droplets are strongly Schiff-positive except in inner fasciculata.

FIG. 9. Rat on pantothenic-acid deficient diet for 1 week (#27). Sudan IV and hematoxylin, photographed as fig. 7. Loss of lipid from innermost zones, reduction in fasciculata, maintenance in glomerulosa.

FIG. 10. Rat on pantothenic-acid deficient diet for 4 weeks (#25). Schiff-plasmal reaction. Photographed as fig. 8. Some hypertrophy of cortex. Further reduction of ketosteroids. Watery vacuolization of outer fasciculata cells.

FIG. 11. Rat on pantothenic acid deficient diet for 6 weeks (#21). Sudan IV and hematoxylin; photographed as figs. 7 and 9. Almost total disappearance of lipid from zona fasciculata. Further hypertrophy of cortex. Continued vacuolization of fasciculata cells.

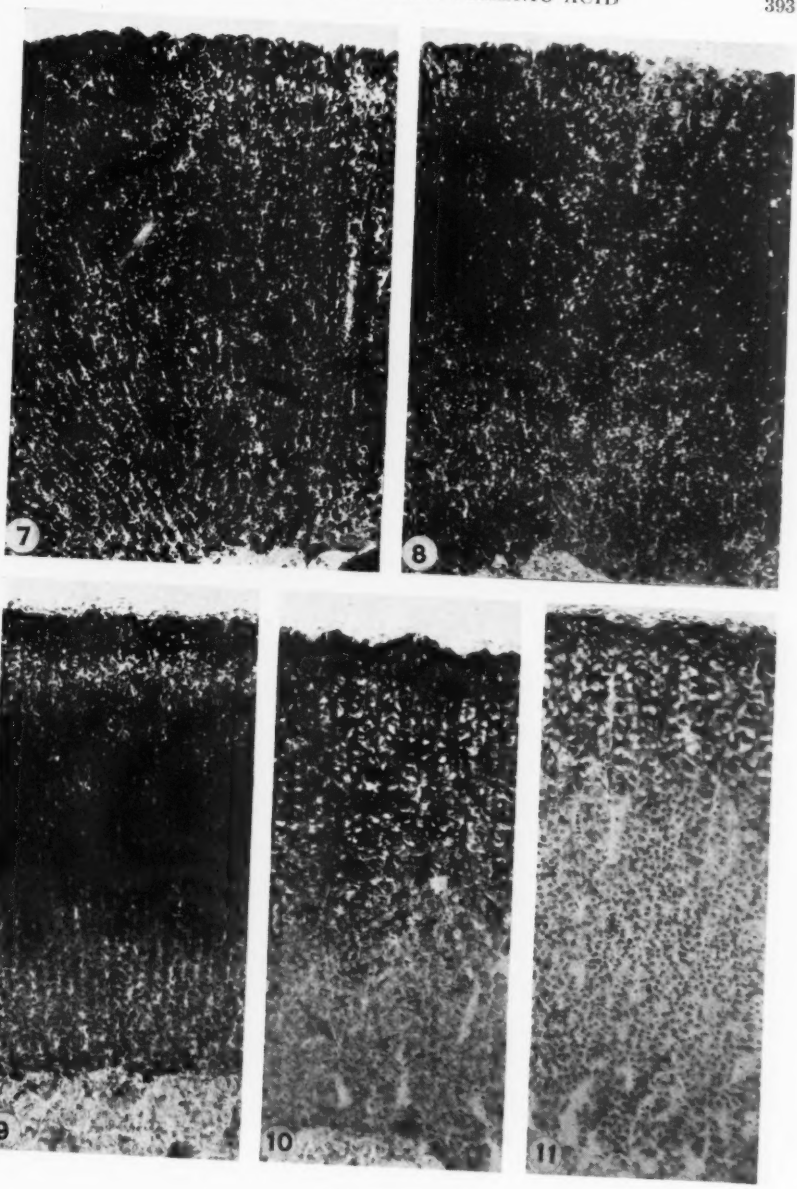

PLATE 1
FIGS. 7–11
(See opposite page for explanation)

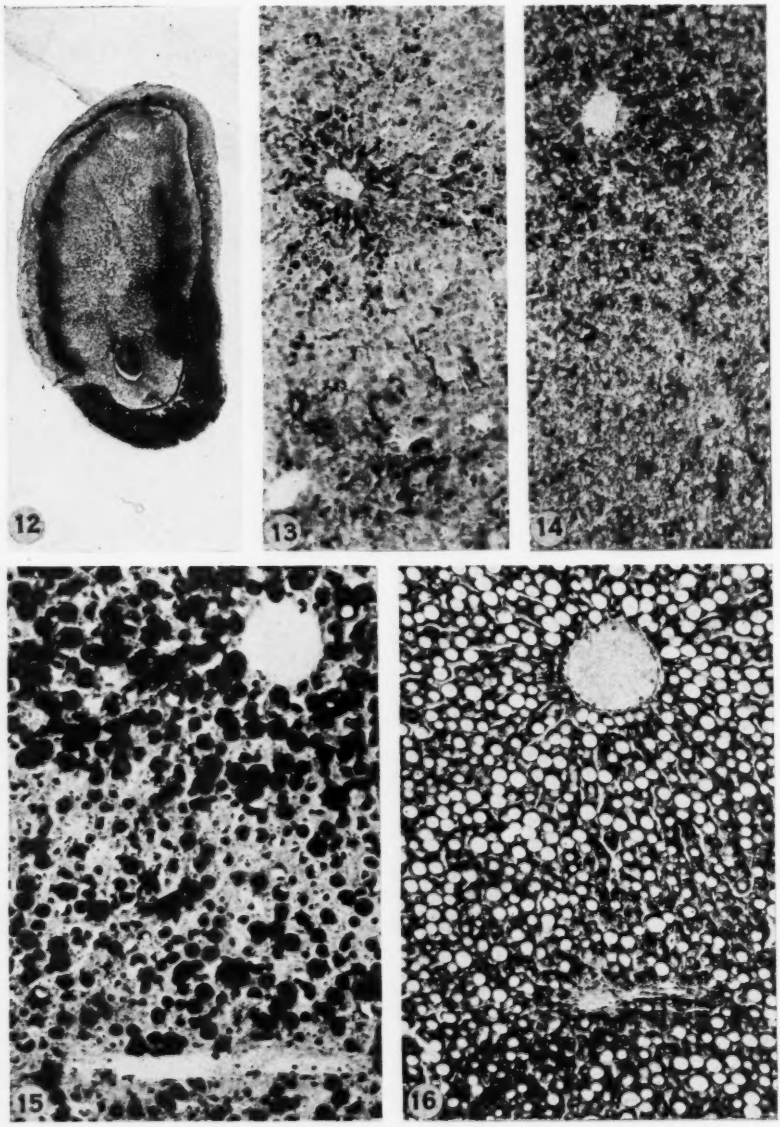

PLATE 2

FIG. 12. Adrenal from rat on pantothenic acid-deficient diet for 5 weeks (#16). Gland fixed in 10% neutral formalin, frozen section cut at 15μ, stained with sudan black B. Photographed with blue filter. Unusual degree of pathological change in adrenal. Black line shows inner limit of recognizable cortical material. Cortical cells inside this line undergoing fatty degeneration. ×24.

FIG. 13. Liver from rat on control diet for 4 weeks (#6), fixed in 10% neutral

tions of a regression of the cells of the zonae fasciculata and reticularis appeared. The cells of these zones contained progressively fewer spaces occupied by lipids, and the mitochondria became altered and greatly reduced in number. In some cells the mitochondria appeared unusually swollen and light staining, while in others they stained darkly and seemed to be fused. Furthermore, after the second week of the deficient diet, the peculiar bodies defined as liposomes disappeared from the cells of the fasciculata.

The kidney. The kidneys of the control rats presented nothing unusual. No lipoid droplets were encountered in the cells of the tubules and the mitochondria of the cells of the proximal and distal tubules formed smooth filaments or batonnets, located at the base of the cell and oriented toward the lumen. Similarly, the kidneys of the deficient animals exhibited no lipoid droplets, but preparations for mitochondria revealed abnormalities after the fourth week of the experiment. These abnormalities consisted in an apparent disintegration of the cells near the kidney medulla in both the proximal and distal tubules, with the cells appearing greatly distended and their mitochondria fragmented and swollen. These changes were observed in all of the rats killed at this time and were dependent upon no specific pathological changes in the adrenals or liver, or in the condition of the animal.

The liver in the control rats. Normally the hepatic cells of the rat contain few or no fat droplets. Consequently, the transient development of acutely fatty livers in the control animals of our series constituted an unexpected occurrence, an explanation of which we will offer subsequently. The livers of control rats killed during the first two weeks of the regimen showed only scattered cells which contained fat droplets, but by the end of the third week there was considerable fat in the cells of the central part of the liver lobule (Figs. 13 and 14). The accumulation of fat reached its peak at 5 weeks. The droplets were initially small, but as they accumulated they fused into a single large drop that filled the cell and gave it the appearance of a typical

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formalin; frozen section cut at 15μ, stained with sudan IV and hematoxylin. Photographed with blue filter. Lipoid droplets numerous around central vein above, sparse near portal canals below. ×105.

FIG. 14. Liver from rat on control diet for 3 weeks (#7), fixed in 10% neutral formalin; frozen section cut at 15μ, stained by the Schiff-plasmal method. Photographed with green and yellow filters combined. Lipid has same distribution as in fig. 14, droplets wholly or in part Schiff-positive. ×105.

FIG. 15. Liver from rat on control diet for 5 weeks (#2), prepared as fig. 13. Single fat drops virtually fill cells, especially near central vein above. Such drops are birefringent, Schiff-positive, and autofluorescent, and thus may be presumed to contain ketosteroids. ×105.

FIG. 16. Liver from rat on control diet for 5 weeks (#2), fixed in picro-alcohol-formalin. Paraffin sections cut at 5μ, stained by the Bauer-Feulgen method for glycogen and counter-stained with hematoxylin. Photographed with yellow and green filters combined. Vacuoles of the large fat drops seen in fig. 15. All cells contain considerable glycogen. ×105.

fat cell (Fig. 15). During the first 2 weeks the droplets were merely sudanophilic in reaction and thus probably triglycerides, but by the third week they also gave to a slight degree the various tests of ketosteroids, i.e., the Schiff plasmal reaction (Fig. 14), birefringence and autofluorescence. It should also be noted that in mitochondrial preparations some droplets were revealed which had stained darkly with the hematoxylin, in this respect resembling the liposomes of the adrenal. Between the fifth and sixth weeks of the period of observation this fat disappeared so that the livers of the last control animals appeared to be entirely normal.

The livers of the control rats, even when excessively fatty, exhibited without exception a great store of glycogen (Fig. 16), although the animals were always killed in the afternoon, which is the time of glycogenolysis (Deuel et al., 1938). Glycogen was distributed evenly in the lobule, as is the case whenever it occurs in the liver in large amounts (Deane, 1944).

In mitochondrial preparations no signs of true cell abnormality were manifest. Even when fatty, the cells contained fine, filamentous mitochondria that appeared entirely normal. On the other hand, occasional dying cells were present in which the mitochondria were apparently dissolved. And mitotic figures were very common in all the livers, a finding which we regard as unusual in rats of this age.

The liver in the deficient rats. In contrast to the livers of rats on the control diet, droplets of sudanophil fat were observed only rarely in the hepatic cells of rats on the pantothenic-acid free diet. This relative lack of fat in the livers of the deficient rats should probably be regarded as a symptom of deficiency. Moreover, in only one instance did this lipid give the ketosteroid reactions (rat #14).

In most of the livers of the deficient animals there was a considerable quantity of stored glycogen. In general it may be said that this amount was less than that stored in the livers of the corresponding controls and also more irregular in its distribution within the lobule. Furthermore, in two livers there was virtually no stored glycogen (#34, 4 weeks; #22, 6 weeks), a finding which bore no obvious relation to other changes in the rats.

The hematoxylin preparations demonstrated the presence of mitoses in the hepatic cells only during the first week of the experiment in contrast to their more extensive occurrence in the controls, and this finding should probably be interpreted as a sign of deficiency. Second, although the mitochondria were, in general, fine and filamentous in form, they frequently stained poorly. This condition was always related to a conspicuous illness of the animal, involving extreme lassitude, excessive porphyrin deposits on the whiskers, very rusty hair, and extreme depletion of the adrenal lipids. Third, it became apparent that the individual hepatic cells of the deficient rats were small in comparison with those of the controls. This smallness

was not due solely to the relative lack of fat, since the nuclei were likewise small. Indeed, the cells appeared shrunken and compact, very much like the cells of the adrenal reticularis. Finally, many more dying cells were observed than in the livers of the controls.

DISCUSSION

Pantothenic acid deficiency as an "alarming" stimulus. The adrenals of rats on a diet markedly deficient in pantothenic acid manifested many of the changes that have beeen described as characteristic of the "alarm reaction" of mammals to conditions of stress (Deanesley, 1931; Selye, 1937; Dalton et al., 1944; Popják, 1944; Sayers et al., 1944). As early as one week after being placed on the deficient diet the rats had adrenals that were larger in proportion to body weight than those of their controls (Fig. 3). There was subsequently a relative hypertrophy of the adrenal cortex which was associated with a disappearance of ketosteroids from the whole inner part of the cortex, first from the reticularis and then from the fasciculata. After five or six weeks on a deficient diet the reactions indicative of ketosteroids had disappeared from the entire cortex excepting the glomerulosa, which itself remained apparently unaffected (Figs. 6, 9, 10, and 11). During the process of depletion small steroid droplets were commonly observed in the lumina of the blood vessels, suggesting an outpouring of hormone. Moreover, hematoxylin preparations revealed not only a disappearance of the liposomes from the fasciculata which, according to Hoerr (1936), are related to the secretory products of the cells, but changes in the mitochondria here as well. The latter became swollen and indistinct, which is the reverse of the changes which they undergo in the fasciculata after hypophysectomy (Deane and Greep, 1946). Concomitantly the inner portion of the zona fasciculata as well as the zona reticularis became more and more distorted, necrotic and frequently hemorrhagic. An unusual number of cells in these zones of the cortex became dark staining (siderophilic), a cytomorphosis which both Bennett (1940) and Wolfensohn (1941) have ascribed to senescence of the cortical cells following secretion. Also significant is the observation of an unusual number of mitotic figures in the outer part of the zona fasciculata of the deficient rats during the first two weeks of the experimental period. Hoerr (1941), Blumenthal and Loeb (1942), and others have described an identical augmentation of mitosis after a number of other "alarming" stimuli, and it may be in part responsible for the observed enlargement of the cortex. Moreover, the deficient rats showed many other characteristics listed by Selye as occurring in the advanced stages of the "alarm reaction," such as lassitude, gastritis, and hemorrhagic thymuses and kidneys.

All of these observations point to a progressive depletion of ketosteroids from the zona fasciculata of the adrenal cortex despite its hypertrophy. The hypertrophy indicates an increased secretion of

adrenal hormone, while the depletion indicates that the production is still inadequate for the demand (Deanesley, 1931; Rokhlina, 1940). According to Sayers et al. (1944), changes as pronounced as these reveal that the "alarm" stimulus is of such severity that the organism cannot tolerate it. Certainly animals on a pantothenic-acid deficient diet ultimately succumb.

Deane and Greep adduced evidence to show that the zona fasciculata is responsible for the production of the corticosterone-like hormones, which govern gluconeogenesis, whereas the zona glomerulosa seems to be the site of formation of the steroids such as desoxycorticosterone, which affect electrolyte balance. The depletion of ketosteroids from the fasciculata and their retention in the glomerulosa therefore indicate augmented secretion of the corticosterone-like substances by the fasciculata in the presence of normal elaboration of desoxycorticosterone-like ones by the glomerulosa.

The disappearance of the ketosteroid reactions from the fasciculata in spite of its hypertrophy was accompanied by other signs of an unusually high level of secretion of corticosterone-like substances. There was an acute involution of the thymus in the deficient animals in contrast to their controls (Fig. 4), and Dougherty and White (1945) have succeeded in correlating involution of lymphoid tissue directly with the amount of secreted corticosterone. The involution began before the end of one week on the deficient diet, a fact which suggests an immediate involvement of the cortex in pantothenic-acid deficiency. It should be noted in passing that the speed of both the thymus involution and the exhaustion of the adrenal cortex exceeded what would be expected from a comparable degree of simple inanition. The deficient animal grew slowly, to be sure, but extreme inanition, involving an actual loss of weight, or complete starvation, is necessary to produce a similar exhaustion of the cortex (Blumenthal and Loeb, 1942; Whitehead, 1942). Moreover, in those animals that had reached an advanced stage of adrenal insufficiency, there was a reduction of stored glycogen in the liver, possibly denoting a decrease in circulating corticosterone at that time and resulting in a reduction of gluconeogenesis (Long, Katzin and Fry, 1940). Gaunt, Liling and Mushett (1946) have recently reported other physiological symptoms of adrenal insufficiency in rats examined after they had developed an extreme deficiency in pantothenic acid.

As mentioned above, changes in the adrenals, liver and thymus similar to those reported here have been observed in mammals following numerous other stress conditions, ranging from injections of formaldehyde to extreme fatigue. In all instances they have been analyzed as the response of the adrenal to pituitary adrenotropin released because of the stress. Since the mechanism by which the pituitary is stimulated to secrete this hormone is unknown, the rôle of pantothenic-acid deficiency is correspondingly obscure. It may be

relevant that in a specific group of bacteria, Dorfman, Berkman and Koser (1942) showed that pantothenic acid is concerned with pyruvic-acid metabolism. This and other observations suggest that pantothenic acid is concerned with carbohydrate metabolism. Perhaps a disruption of the normal pattern of carbohydrate metabolism in the tissues could stimulate the pituitary in some way. Moreover, deficiencies in other water-soluble vitamins (e.g., ascorbic acid, riboflavin, or pyridoxine), also concerned with carbohydrate metabolism, produce many of the same symptoms, so that the stimulation of the pituitary may not be a result of pantothenic acid deficiency specifically.

Fatty liver on the control purified diet. Despite the fact that the control rats of this experiment grew quite normally and revealed no signs of adrenal stimulation, their livers became infiltrated with a large amount of lipid, some of which was ketosteroid in nature. The fat droplets were unusual in that they were generally single and caused the hepatic cells to resemble ordinary adipose cells. Such large fat droplets are rare in the liver. However, the fatty state of the hepatic cells in our rats was transitory since by the end of six weeks the livers were almost fat-free. Also, besides the transitory feature of the fat accumulations, the hepatic cells remained quite normal in most respects; thus they appeared to be normal in regard to cell size and nuclear form, the complement of mitochondria and the storage of glycogen. Nevertheless, an unusual number of them did undergo cell division, and some were dying. It would seem desirable, therefore, to study the purified ration employed in this study further to learn why it caused this unusual accumulation of fat in the liver. The ration is deficient in folic acid, biotin, and perhaps other water soluble vitamins which may be important in the nutrition of the rat. Ordinarily these factors are furnished by bacterial synthesis in the intestine in quantities adequate for growth. It is possible that this synthesis may have been temporarily disturbed by the purified diet.[2] The genesis of the observed ketosteroid component of this liver fat is unknown.

SUMMARY AND CONCLUSIONS

Weanling male rats placed on a purified diet deficient in pantothenic acid grew poorly in comparison to their controls and presented other symptoms commonly described for this deficiency. Moreover their adrenal cortices became relatively large and the zonae reticularis and fasciculata were progressively drained of ketosteroids and gave cytological evidence of exhaustion. By the end of six weeks the zona fasciculata was entirely depleted of its hormone. While there was no

[2] Experiments completed since this paper went to press confirm this suggestion since the addition of p-aminobenzoic acid, biotin, and inositol to the control purified ration almost completely prevented the infiltration of the liver cells with fat (Deane & Shaw, unpublished).

apparent significant alterations in blood glucose and non-protein nitrogen, the severely deficient animals exhibited a decrease in liver glycogen as well as shrunken and often dying liver cells. After the fourth week of the deficiency the kidney tubule cells near the medulla likewise showed signs of degeneration. The thymuses of the deficient rats were atrophied in comparison to those of the controls. All of these symptoms are interpreted as indications that a pantothenic acid deficiency acts as an "alarming agent" for the rat. Such agents cause the release of pituitary adrenotropin which stimulates the adrenal to enlarge and increase its secretion of corticosterone. A deficiency of pantothenic acid is so severe an "alarming agent" that the adrenal becomes exhausted of corticosterone.

The livers of the control animals exhibited a transitory fatty infiltration which is tentatively attributed to a deficiency of certain water soluble vitamine ordinarily provided by bacterial synthesis in the intestine.

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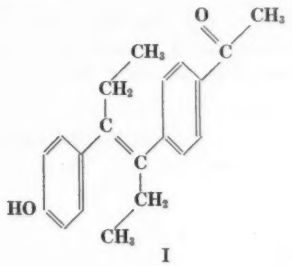
NOTES AND COMMENTS

CHEMICAL STRUCTURE AND PROGESTERONE-LIKE ACTIVITY

THE NOTIONS developed in the article "Sex Hormonal Action and Chemical Constitution" (Schueler, 1946) for dealing with the structural and chemical characteristics a given substance must possess to elicit male or fe-



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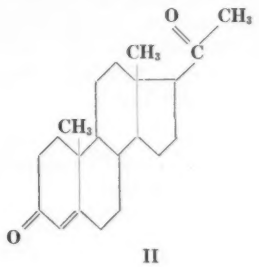

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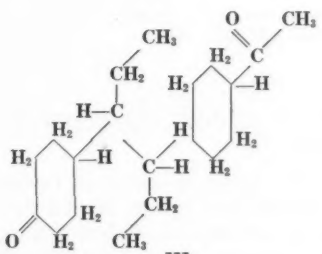

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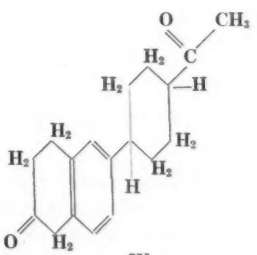

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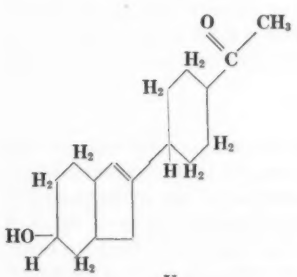

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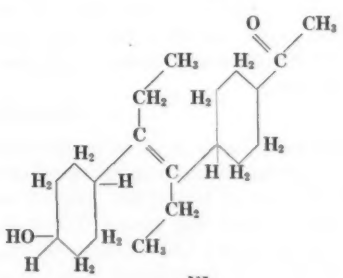

VI

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male sex hormonal activity have been extended, in an attempt to include other biologically active substances related to the steroid hormones.

In particular, work is now in progress which may make the synthesis of non-steroid progesterone-like compounds possible. Early work by Dodds (1936) indicated that substances built on the α,β diethyl-stilbene nucleus might show progesterone-like activity. Formulae I and II show the structural relationship of a stilbene derivative to progesterone.

In the light of the differences in hydrogen bond forming power of aromatic -OH groups and secondary alcoholic -OH groups, substance I would be expected to have but weak progesterone-like properties, if any. Also, since the likelihood of the hydrolytic removal of the acetyl group in I might be expected during *in vivo* processes, any progesterone-like activity it might possess would be masked by the estrogenic response of its possible hydrolytic product diethyl stilbestrol (R. Courrier *et al.*, 1943). That this is probably the case has been borne out by experiment.

For these reasons, the suggestion is here put forth that the synthesis of non-steroid progesterone-like substances be attempted through the utilization of saturated or partially saturated rather than aromatic ring systems, e.g. III, IV, V, and VI.

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THE TOXICITY OF THE SERUM OF ADRENALECTOMIZED ANIMALS

In 1936 Riml reported the presence of a toxic substance in the blood serum of adrenalectomized rabbits. He found that serum or dialysate of serum taken from rabbits showing signs of adrenal insufficiency three or four days after removal of the adrenals was lethal when injected into guinea pigs; while serum or serum dialysate from intact rabbits had no untoward effect. Since the nature of the toxic substance has not been determined and since no confirmation of Riml's work has been reported, we decided to reopen the problem.

Experimental

Rabbits and cats were adrenalectomized in two stages. Adrenal extract (5 cc. of Upjohn's) was administered after the removal of the second adrenal for the purpose of tiding the animals over the effects of the operation, but no further extract was given. When the animals showed signs of marked adrenal insufficiency (13–46 days postoperatively), they were bled by heart puncture. Five cc. samples of blood were taken for plasma electrolyte determinations, the results (decreased sodium and increased potassium) of which

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further showed that the animals were in adrenal insufficiency (Baumann and Kurland, 1926–27). The blood was allowed to clot, the serum was collected and some of the serum was dialyzed against an equal amount of normal saline in the cold. An intraperitoneal injection of from 2 to 20 cc. of serum or serum dialysate was given to rats and guinea pigs. Since the injections did not cause death, the animals were sacrificed 48 hours later and the adrenals were removed and weighed. The adrenal weights of the treated animals were found to be no different from those of untreated controls. Riml reported an increase in weight of the adrenals as a result of the administration of serum or dialysate of serum from adrenal insufficient rabbits.

Thus we were unable to confirm the report of Riml that the serum and serum dialysate of adrenalectomized rabbits is either lethal to normal guinea pigs or causes an increase in the weight of their adrenals. A possible basis for the discrepancy in results might be found in the failure of Riml to administer adrenal extract for the purpose of permitting the animals to recover from the effects of the operation. Perhaps the lethal substance in his experiments arose as a result of the operative shock rather than as a result of the development of adrenal insufficiency.

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NEW BOOK

ANNUAL REVIEW OF PHYSIOLOGY. Vol. VIII. By James Murray Luck, Editor. $5.00. viii+658 pp. Annual Reviews, Inc. 1946.

A cover-to-cover reading of this year's *Annual Review of Physiology* leaves a man with rather more data then he knows what to do with. Perhaps it is only natural that this surfeit makes one wish that some of the reviewers had been more selective of the intellectual food to lay before one and more attentive to its preparation. A few of the reviews seem to be mere lists of observations, each carefully attributed to the original author but with no mention of relevance to existing hypothesis or even of consistency or inconsistency with previous information. Fortunately, however, the majority of authors have recognized that their primary task is to present the recent progress in their field in such a way that its significance may be grasped by intelligent outsiders. Rarely, as in the case of Bishop's article on nerve, wit and wisdom combine to amuse as well as edify.

Once the process of ingestion is over and assimilation fairly begun it may be worth while to reflect a little on what these reviews reveal about the field of physiology as a whole. One interesting observation, for instance, is the fact that the majority of reviews were written by persons not themselves professional physiologists. Anatomists as usual justify their proprietorship of the field of endocrinology with excellent reviews of "metabolic functions" and reproduction. A psychologist demonstrates that the integrative action of the nervous system is not to be left much longer in the hands of classical physiology, if indeed it has not already left them.

If anything, this centrifugal tendency is even more clear in the papers with which the reviews deal. Broadly speaking, physiological investigation appears to be concerned with two sorts of subjects—the intimate mechanisms of cellular metabolism and the coordination or integration of these mechanisms to produce adaptive behavior. Work of the first type seems to fall increasingly in the domain of biochemistry where hardly a day goes by without the announcement of a new enzyme, coenzyme or antiesterase. Problems of integration distribute themselves among various disciplines. Recent advances in cardiovascular physiology have come largely from clinical medicine armed by Cournand with ten-foot venous catheters and the Fick principle; the anatomist has manfully held onto the early lead given him in endocrinology by his command of the microscopic changes wrought by hormones, but biochemists and immunologists are increasingly joining the field. To judge from the current reviews it now seems apparent that Selye's alarm reaction filled in and refined by the discoveries of White and Dougherty and Houdack and McMasters constitutes the most important contribution to the field of adaptive physiology since Cannon's hypothesis of the homeostatic function of the sympathetic system. And not one of these contributors is classified, academically at least, as a physiologist.

The nervous system still appears to attract many professional physiologists but it is by no means clear that adherence to the time-honored methods

of ablation, stimulation and electrical recording are going to provide answers to the questions we really wish to ask. The dissection of behavior into unit reflexes, each with its discrete anatomical site, has been extremely helpful, especially to the neurosurgeon and clinical neurologist. Indeed it is a clinician who reviews the somatic aspects of neurophysiology in the present volume. Most of the recent work may, however, be included in Bishop's indictment of his own review as failing "to present the forest because its assignment is so specifically to examine the trees." One wonders why physiology, which has in the past claimed such notable foresters as Harvey and Claude Bernard, is currently so complacent about leaving the synthetic aspects of its job to others.

Definitive evaluation of the contribution of "war research" to basic physiological knowledge is still brass bound. Judging from the many references made by reviewers, desks in Washington are piled high with military secrets ranging from the diffusion coefficient of oxygen to preventatives for sunburn. It seems clear, however, that knowledge of respiratory physiology has been refined and enlarged, and the several elements composing the picture of surgical shock more precisely evaluated. Detailed directions are available for what clothes to wear, what food to eat, and what equipment to take along on trips to every part of the globe and into the air for at least 40,000 feet above it.

Civilian work in basic areas has managed to continue and provides some points from which to view the future. An admirable review of the physiology of the skin promises that this important organ may soon receive as intelligent medical attention as that provided for more inaccessible systems. Here and there throughout several of the reviews there are broad hints that our knowledge of cellular physiology, genetics and growth is being greatly enlarged by application of refined physical and chemical methods. Both articles on the endocrine system emphasize the need for investigations of target organ sensitivity in explaining obscure and anomalous reactions to hormones.

In summary, it is a pleasure to report that in spite of the many difficulties of the last several years biological science has continued to advance and make plans for an uncertain future. The editors and contributors of the present volume of Annual Review of Physiology stole time and energy from other pressing duties to lay the record before us. They will have the gratitude of men returning to laboratories throughout the world, many for the first time in seven years, to catch up with the past and work out the future of biology.

ROBERT S. MORISON

ASSOCIATION NOTICE

SQUIBB AND CIBA AWARDS
ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Association for the Study of Internal Secretions has just announced that the award furnished by E. R. Squibb and Sons has been given to Dr. Carl G. Hartman of the University of Illinois and that the award furnished by the Ciba Pharmaceutical Products, Inc., has been given to Dr. Martin M. Hoffman of McGill University.

The Squibb award to Dr. Hartman was based on his fundamental contributions to the physiology of reproduction and the hormonal factors involved in menstruation as well as his fundamental observations on the behavior of insects. Dr. Hartman is Professor of Zoology and head of the Departments of Zoology and Physiology at the University of Illinois. He received his degree of Master of Arts from the University of Texas in 1904 and the degree of Doctor of Philosophy from the same institution in 1915. He was first an instructor and later Professor of Zoology at the University of Texas, from 1912 to 1925; and then a Research Associate in Embryology at the Carnegie Institute from 1925 to 1941, following which he assumed his present duties at the University of Illinois.

The Ciba award to Dr. Hoffman was based on his observations on the metabolism of oestrogens and progestrone as well as those on the excretion of cortin in the urine following trauma. Dr. Hoffman received his degree of Doctor of Philosophy in biochemistry at McGill University a few years ago, and since then has worked as a fellow in the Laboratories of Dr. J. S. L. Browne at Royal Victoria Hospital in Montreal, Quebec, Canada. He is, at the present time, completing his requirements for the degree of Doctor of Medicine. He was only 29 years old at the time the award was made.

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*Greene, R. R.; Int. Abst. Surg. 74: 595, 1942

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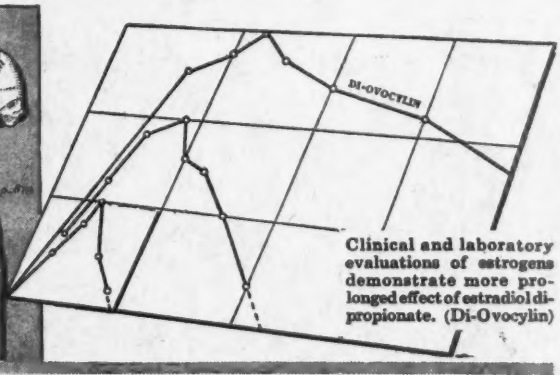

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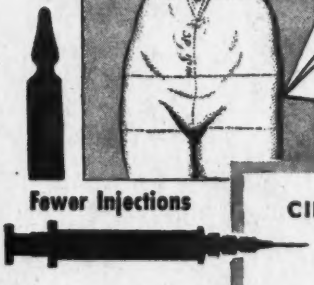

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